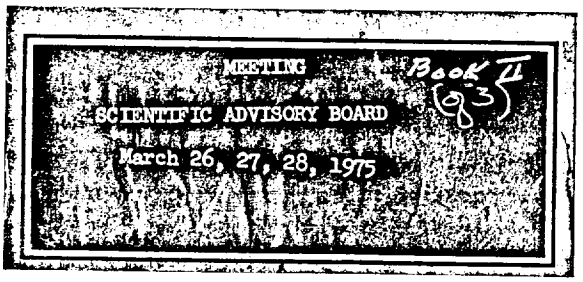

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PULMONARY

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#976M - AYRES

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

February 5, 1975

Grant Application No. 976M

PULMONARY

To: The Committee comprising Drs. Gardner, Liebow and Wyatt

Subject: Stephen M. Ayres, M.C., Saint Louis University,
Saint Louis, Missouri

New Application No. 976M
"Relationship of Genetic Predisposition, Exposure to High Level Automotive Pollution and Cigarette Smoking to the Development of Chronic Airways Obstruction."

History

Grant application No. 976 (made when Dr. Ayres was located at St. Vincent Hospital, Worcester, Mass.) was deferred at the March 1974 SAB meeting, for further information and site-visit.

At the October-November SAB meeting, the application was disapproved in its original form.

The present application No. 976M is a modification of the original application.

Request

Application No. 976M requests $41,611 for the first year of a 3 year program.

Documents Submitted (attached)

1. Application, received by CTR February 5, 1975 (11 pages, including CV of Dr. Ayres).

DS:jmb
Enclosures

D.S.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

#1764

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 5 1975

Application for Research Grant

(Use extra pages as needed)

Date:

1. Principal Investigator (give title and degrees):
Stephen M. Ayres, M.D.
Chairman, Department of Internal Medicine

2. Institution & address:
Saint Louis University
1325 South Grand Boulevard
Saint Louis, Missouri 63104

3. Department(s) where research will be done or collaboration provided:
Medicine

4. Short title of study:
Relationship of Genetic Predisposition, Exposure to High Level Automotive Pollution and Cigarette Smoking to the Development of Chronic Airways Obstruction.

5. Proposed starting date:

6. Estimated time to complete:

7. Brief description of specific research aims:
We plan to evaluate the relationship of three specific risk factors to the development of chronic airways obstruction. The prevalence of predisposing factors and cigarette smoking will be determined in 200 men who have been exposed to high concentrations of automotive pollution for a number of years. Small airway disease will be identified by symptom inventory, measurement of closing volume, and measurement of expiratory flow-volume curves breathing room air and helium. Emphysema will be identified by symptomatology, evaluation of chest radiography, measurement of total lung capacity, and determination of single breath diffusing capacity for carbon monoxide.

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8. Brief statement of working hypothesis: (continued on attached page)

This study seeks answers to the following questions:

Are any or all of the three risk factors related statistically to the development of small airways disease or emphysema?

Are the factors additive or synergistic? Can a table be constructed showing the prevalence of either small airways disease or emphysema with no, one, two, or three risk factors present?

What is the time frame for the development of either small airways disease or emphysema in relationship to onset of initial exposure?

Does small airways disease always progress to emphysema? Does emphysema always follow a period of small airways disease?

Are there specific heterozygous phenotypes determining alpha one antitrypsin concentrations which are related to the development of chronic airways obstruction?

9. Details of experimental design and procedures (append extra pages as necessary)

We have performed yearly studies on over 450 bridge and tunnel officers over the past three years under contract from the Triborough Bridge and Tunnel Authority. This population group is composed of men who collect tolls and control traffic for seven bridges and two tunnels in New York City. These men are exposed to extremely high concentrations of automotive emissions and have high blood carboxyhemoglobin levels. Our study was begun at the request of both Management and Union in order to determine the prevalence of cardiac and pulmonary disease in the population at risk.

An appended reprint details the first two years of experience. Over 75% of the men had laboratory evidence of small airways disease. Figure 1, accompanying this report, shows a Venn diagram depicting the distribution of laboratory and historical abnormalities. Neither detailed smoking or family histories were obtained, but a similar incidence of pulmonary abnormalities were observed in both smokers and non-smokers. In this group, at least environmental pollution rather than cigarette smoking appeared to determine the development of chronic airways obstruction.

The principal investigator will be moving to Saint Louis University School of Medicine as Chairman of Internal Medicine in July 1975. Saint Louis University has traditionally been interested in the determinants of chronic airways obstruction so that the interest of our group in New York City will coincide with established research interests in Saint Louis. Dr. G. O. Broun, Sr. has monitored the importance of viral illnesses to the progression of chronic airways obstruction, and members of the Department of Pathology have contributed to an understanding of the morphology over a period of many years. The availability of expertise in biochemistry, immunology, and the behavioral sciences will improve our ability to achieve the objectives developed in this proposal.

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8. Cont'd.

Is carcinoma of the lung related to genetic and environmental factors as well as to cigarette smoking?

9. Cont'd.

Almost 200 of the 450 bridge and tunnel workers were studied comprehensively during 1972 and 1973. Alveolar carbon monoxide determinations and screening spirometry were performed on-site in the various work locations. They then visited our laboratory facilities on West 12th Street in Manhattan for detailed evaluation. The evaluation included completion of the Cornell Index, completion of a special questionnaire on pulmonary symptomatology based on the British Medical Research Council Questionnaire, physical examination, chest radiography, electrocardiography, clinical laboratory study and pulmonary function study. The pulmonary function evaluation included spirometry, measurement of expiratory flow-volume curves, body plethysmography, measurement of closing volumes and nitrogen washout.

The cohort of 200 individuals form the study group for the proposed research project. We propose to follow their pulmonary function at yearly intervals and attempt to evaluate the prevalence of the risk factors enumerated above.

Pulmonary Function Evaluation: This will be continued on a yearly basis in a manner identical to that performed on the first visit. In addition, flow-volume curves while breathing helium will be obtained because of the recent demonstration that such curves may identify early evidence of small airways disease. Thus far, we have relied on measurements of functional residual capacity and nitrogen washouts to estimate the degree of emphysema. The steady state diffusing capacity for carbon monoxide has been shown to reflect the degree of emphysema and we plan to add this to the yearly evaluation.

Estimation of Exposure to Automotive Pollution: This will be based on the cumulative data of both blood and alveolar carbon monoxide measurements made on-site since the beginning of this study three years ago. Job history, (tunnel or bridge, maintenance, office work) will also be used to evaluate air pollution exposure.

Estimation of Smoking History: A detailed smoking history will be obtained on each individual at the time of initial retesting. This history, together with the Predisposition History described below will be gathered using a personal interview technique. Miss Felicity Reimold has participated in studies of this nature for the past five years and will collect and record the information. The history will include pack-years, type of cigarette smoked, onset of smoking, discontinuation of smoking and years of abstinence if applicable.

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9. Cont'd.

Estimation of Genetic Predisposition: Family history will be evaluated at the time of initial retesting. Specifically, we will ask questions relating to history of pulmonary disease in any family member, history of allergy, tuberculosis, or liver disease.

The prevalence of homozygous or intermediate alpha one anti-trypsin deficiency will be identified by immunodiffusion. This will be performed by Dr. Joseph McKenna at Saint Vincent Hospital and Medical Center in New York City.

Since a key objective of this proposal is to evaluate known biochemical determinants of chronic airways obstruction, subjects with low concentrations of alpha one antitrypsin on immunodiffusion will be studied by starch gel electrophoresis and antigen-antibody crossed electrophoresis to determine phenotype. The laboratory of Dr. John Pierce at Washington University in Saint Louis has had considerable experience with this technique. One of his associates, Dr. Edith Levine will be joining the faculty at Saint Louis University and will assist in the development of this techique.

Supervision of Project: Dr. Darline D. Smith will serve as on-site co-ordinator for the project. She has been responsible for the physical examination and laboratory evaluation for the past year.

Dr. Rodney Coe, Professor of Community Medicine at Saint Louis University and a medical sociologist, will assist in the design and evaluation of the family history and smoking questionnaires. Dr. Lewis Kuller, Chairman of the Department of Epidemiology, University of Pittsburgh, has participated in the design of the entire project.

Data Evaluation: All data is recorded when obtained on data cards designed for the Hewlett-Packard electronic computation system. The storage, retrieval, and evaluation techniques were designed by Mr. Robert Evans who will be responsible for continued processing of data generated by the proposed project.

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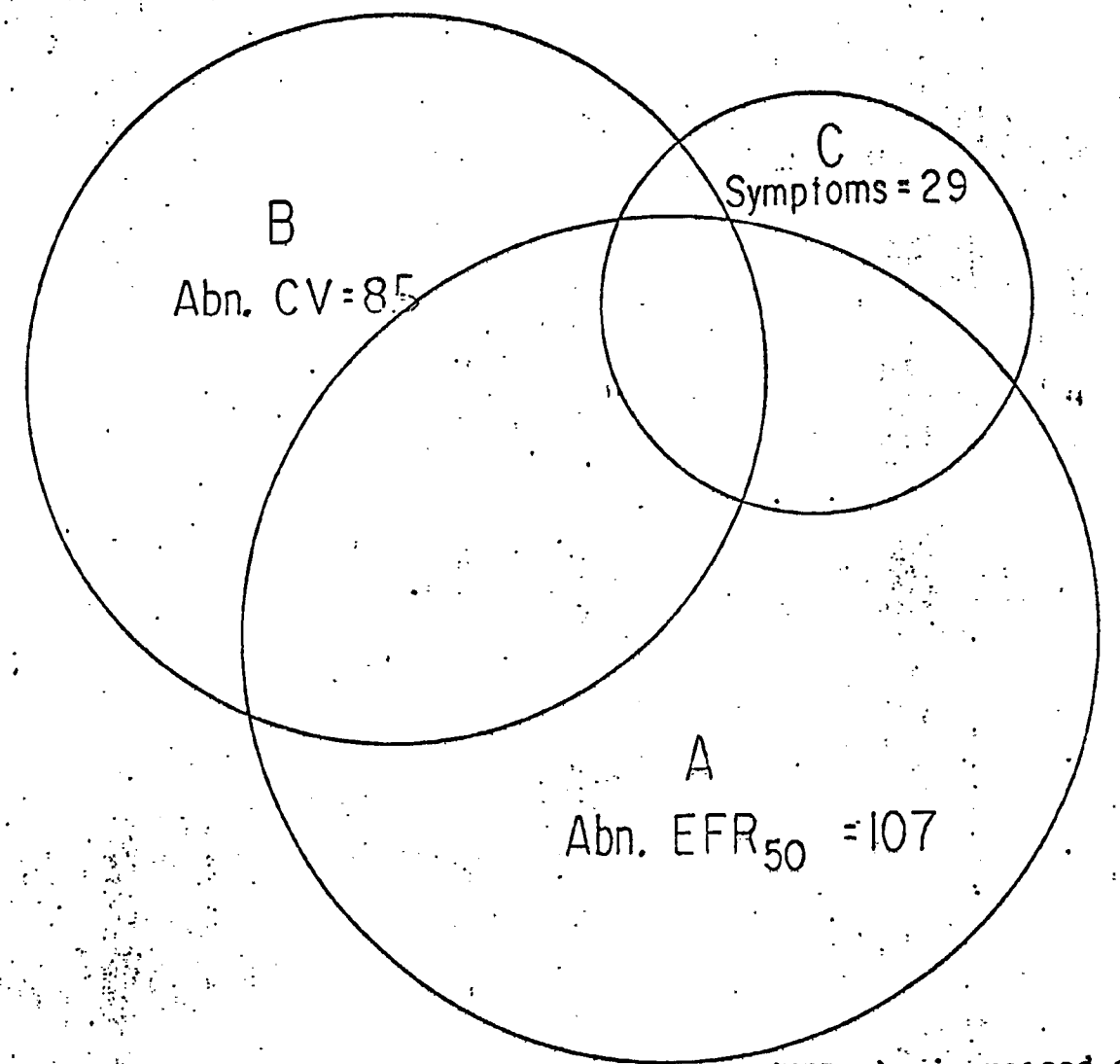

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Figure 1 - Distribution of abnormally low mid-expiratory flow rates (EFR_{50}), increased closing volumes (CV) and symptoms of chronic bronchitis in a population of 111 non-smoking [illegible] and tunnel workers.

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Pulmonary Function Laboratory
117 West 12th Street
New York, New York 10011

Dr. J. McKenna
Immunology Laboratory
St. Vincent's Hospital and Medical Center
of New York
153 West 11th Street
New York, New York 10011

Computer facilities
117 West 12th Street
New York, New York 10011

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Please see attached page.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Please see attached page.

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R: REDACTED MATERIAL

CURRICULUM VITAE

Stephen M. Ayres, M.D.

1929	REDACTED REDACTED
	B.A., Gettysburg College, Gettysburg, Pennsylvania
REDACTED	M.D., Cornell University College of Medicine, New York City
1955	REDACTED
1955-1956	Intern in Medicine, New York Hospital-Cornell Medical Center, New York City
1956-1958	Medical Officer, United States Army
1958-1959	Assistant Resident in Medicine, New York Hospital-Cornell Medical Center, New York City
1959-1960	National Heart Institute Research Fellow, Cardiopulmonary Laboratory, New York Hospital-Cornell Medical Center, New York City
1960-1961	Resident in Cardiology, New York Hospital-Cornell Medical Center, New York City
1961-1963	Director, Cardiopulmonary Laboratory, St. Michael's Hospital, Newark, New Jersey
1961-1963	Clinical Assistant Professor of Medicine, Seton Hall Medical School
1963	Certified, American Board of Internal Medicine
1963-1973	Director, Cardiopulmonary Laboratory, St. Vincent's Hospital and Medical Center of New York, New York City
1964-1968	Assistant Professor of Clinical Medicine, New York University School of Medicine, New York City
1968-1973	Associate Professor of Clinical Medicine, New York University School of Medicine, New York City
1969-1973	Associate Attending in Department of Medicine, New York University Medical Center, New York City
1971	Elected, American Society of Clinical Investigation
1973-75	Physician-in-Chief, Department of Medicine, The St. Vincent Hospital of Worcester, Worcester, Massachusetts
1973-75	Professor of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts
1975-	Professor and Chairman, Department of Internal Medicine, Saint Louis University, Saint Louis, Missouri

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Stephen M. Ayres, M.D. Bibliography

Societies:

REDACTED

REDACTED

REDACTED

Past Appointments:

Member, Medical Advisory Committee, New York City Department of Air Pollution Control
Chairman of Medical Affairs Committee, New Jersey Tuberculosis and Respiratory Disease Association, Inc.
Member, PHS Select Review Committee on Carbon Monoxide, Bureau of Occupation Safety and Health
Board of Directors, Tuberculosis and Respiratory Disease Association of Central New Jersey

Appointments:

Member, Environmental Control Board, New York City (Appointed by Mayor Lindsay)
Member, Mayor's Council on Environment
Board of Directors, National Tuberculosis and Respiratory Disease Association
Chairman, Anti-Smoking Committee
Member, Committee for Guidance of the Respiratory Disease Program
Member, U.S. Surgeon General's Subcommittee on Cardiovascular Aspects of Smoking and Health
Chairman, Committee on Environmental Quality, Medical Society of the State of New York
Member, Medical Advisory Board, American Association for Inhalation Therapy
Chairman, Research Committee of St. Vincent's Hospital and Medical Center of New York, New York City
Board of Directors, Citizens for Clean Air, Inc., New York City
Member, Subcommittee on Environment and Pollution of the Coordinating Council of the Metropolitan Medical Societies
Board of Directors, American Lung Association of Massachusetts

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13. Publications:

1. Ayres, Stephen M., and Buehler, Meta E.: The effects of urban air pollution of health. Clinical Pharmacology and Therapeutics, Vol. 11, No. 3, May-June 1970.

2. Ayres, Stephen M., Giannelli, Stanley, Jr., and Mueller, Hiltrud: Myocardial and systemic responses to carboxyhemoglobin. Annals of The New York Academy of Sciences, Vol. 174, Article 1, October 1970.

3. Ayres, Stephen M., Giannelli, Stanley, Jr., and Mueller, Hiltrud: Carboxyhemoglobin and the access to oxygen. An example of human counterevolution. Archives of Environmental Health, Vol. 26, January 1973.

4. Ayres, Stephen M., Evans, Robert, Licht, David, Griesbach, Jane, Reimold, Felicity, Ferrand, Edward F., and Criscitiello, Antoinette: Health effects of exposure to high concentrations of automotive emissions. Studies in bridge and tunnel workers in New York City. Archives of Environmental Health, Vol. 27, September 1973.

5. Ayres, Stephen M., Giannelli, Stanley, Jr., Mueller, Hiltrud, and Criscitiello, Antoinette: Myocardial and systemic vascular responses to low concentrations of carboxyhemoglobin. Annals of Clinical Laboratory Science, Vol. 3, No. 6, 1973.

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4.

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Dr. Stephen M. Ayres	5%	R
Dr. Darline D. Smith	10%	
Consultations		
Dr. Rodney Coe		R
Dr. Lewis Kuller		
Technical		
Lab Technician	100%	REDACTED
Computer Technician and Data Analyzer	100%	
Sub-Total for A		REDACTED
B. Consumable supplies (by major categories)		
Glassware, gases, chemicals, recording paper and cards	$3,000	
Questionnaire	500	
Sub-Total for B		3,500
C. Other expenses (itemize)		
Reprints and publishing	$ 500	
Computer	1,000	
Travel	1,000	
Sub-Total for C		2,500
Running Total of A + B + C		33,140
D. Permanent equipment (itemize)		
Starch gel and crossed electrophoresis apparatus		3,500
Sub-Total for D		36,640
E. Indirect costs (16% of A-B-C)		4,971
Total request		41,611

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	$4,000	$3,000	0	$5,528	$42,382
Year 3		4,000	3,000	0	5,976	45,815

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Health Surveillance	Triborough Bridge and Tunnel Authority	$70,000	1/71-7/77

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Stephen M. Ayres, M.D.

Signature _____ Date _____

Telephone _____ REDACTED _____
Area Code Number Extension

Checks payable to

Mailing address for checks

Responsible officer of institution

Typed Name _____

Title _____

Signature _____ Date _____

Telephone _____
Area Code Number Extension

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#764D - COCHRANE

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

February 4, 1975

Grant Application No. 764D

PULMONARY

To: The committee comprising Drs. Gardner, Liebow and Wyatt

Subject: Charles G. Cochrane, M.D., Scripps Clinic and Research Foundation
Continuation Application No. 764D
"The Mediation of Inflammatory Injury of Tissue."

History

CTR has supported this study since 1970. In March 1974 the SAB approved a one-year continuation grant (No. 764C) in the amount of $16,560 for the period July 1, 1974 through June 30, 1975.

Request

Application No. 764D requests $25,818 for a one year program.

Documents Submitted (attached):

1. Application dated January 24, 1975 (21 pages, including CV's of Drs. Cochrane, Henson, Russell, Ulevitch, and Kravis, and Progress Report).

2. Four reprints (with acknowledgment to CTR).

3. One chapter (with acknowledgment to CTR).

DS:jmb
Enclosures

D.S.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: Jan. 24, 1975

Continuation Grant

1. Principal Investigator (give title and degrees):

Charles G. Cochrane, M. D. - Member

2. Institution & address:

Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Department(s) where research will be done or collaboration provided:

Department of Immunopathology

4. Short title of study:

The Mediation of Inflammatory Injury of Tissue

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: July 1, 1976

7. Brief description of specific research aims:

1. To study the participation of the kinin forming, intrinsic clotting and fibrinolytic systems in inflammatory tissue injury.
   a. Purification of component proteins
   b. Analysis of mechanisms of activation in vitro
   c. Analysis of their participation in pulmonary disease
2. To study the participation of cellular factors in inflammatory injury

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

8. Brief statement of working hypothesis:

Inflammatory injury, induced by a wide variety of stimuli, is effected by a series of humoral and cellular mediators. While in many instances the causative inciting agents remain unknown, the mediation systems of the injury are common to them all and subject to analysis. Successful therapeutic resolution of inflammatory injury will only come about through careful analysis.

9. Details of experimental design and procedures (append extra pages as necessary)

I. Summary of proposals from original application.

A. The participation of the Hageman factor pathways in the pathogenesis of inflammatory tissue injury.

One of the most important and yet unknown areas in the pathogenesis of inflammatory disease lies in the kinin forming, intrinsic clotting clotting and fibrinolytic systems, otherwise known as the Hageman factor pathways. These systems are outlined in Table I

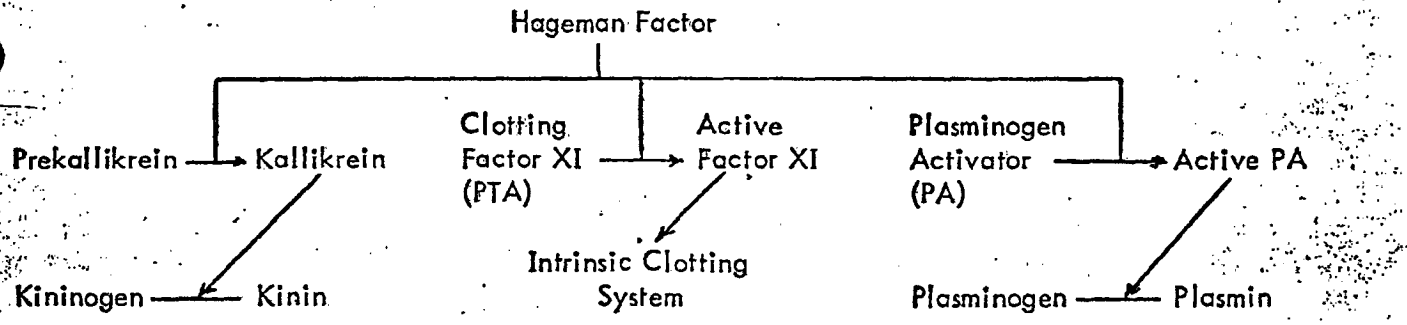

Table I

Activation of these systems otherwise known as the Hageman factor pathways leads to the development of many aspects of the inflammatory process:

1. The injection of activated Hageman factor intradermally induces increased vascular permeability and an influx of inflammatory cells.

2. Kinins cause an increase in vascular permeability and hypotension.

3. Fibrin formation can lead to permanent loss of function and may well stimulate formation of collagen and fibrosis (preventing fibrin deposition in glomerulonephritis blocks fibrosis of the glomeruli).

Source: https://www.industrydocuments.ucsf.edu/docs/mgdm0000

4. Fibrin and fibrinogen degradation products, generated by the action of plasmin, increase vascular permeability and are chemotactic to neutrophils.

5. Plasmin activates C1s leading to stimulation of the complement system.

6. Thrombin, a product of the intrinsic clotting system, can generate activity of C3.

We have devoted five years to the elucidation of these systems by isolating and characterizing components of each in precursor form, defining their mechanisms of interaction and developing methods by which their participation in inflammatory diseases can be evaluated. These studies, which have been supported by the Council for Tobacco Research, have been performed with both rabbit and human plasma. We have chosen rabbits since this species serves well for experimental studies in vivo. With human components, studies can be conducted in clinical disease states.

To date we have been able to isolate in purified form Hageman factor (HF), prekallikrein, factor XI, and plasminogen, from each species. Plasminogen activator has been prepared in impure form. Each protein has been radiolabelled successfully and antibodies prepared to almost all. The activation of HF has received considerable attention and two mechanisms have been defined: solid-phase activation, i.e., that occurring when HF interacts with a negatively charged surface, and fluid-phase activation, i.e., that occurring when enzymes in the plasma, notably kallikrein, act upon fresh HF. These studies have formed the basis of several publications (1,2,3,4). Further data are given in the section on Progress Report.

Continuing experiments, given in detail in the original report, are designed to examine the means by which the HF pathways, i.e., the kinin-forming, intrinsic clotting, and fibrinolytic systems participate in inflammatory tissue injury. In brief, these studies will be conducted as follows:

1. Studies of the participation of the HF pathway components in inflammatory pulmonary disease (see below) by:

   a) Determination of turnover times of ^{125}I labelled components
   b) Changes in levels of components in plasma during the development of injury
   c) Detection of activated components in plasma

2. Detection of activated components bound at the site of injury using fluorescent antibodies and ^{125}I labelled components.

3. Assessment of the effect of deprivation or specific inhibition of activated components on the development of experimental inflammatory lesions.

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Several means instituting inflammatory injury of the lung will be employed:

1) Antibodies directed to pulmonary membrane infused intravenously; 2) repeated inhalation of vaporized soluble antigen into rabbits previously immunized; 3) inhalation of fine particulate kaolin and antigen bound to kaolin in a previously immunized rabbit; 4) repeated intravenous administration of soluble antigen-antibody complexes and bacterial lipopolysaccharide. In each type of injury, acute inflammation results, with influx of inflammatory cells and formation of fibrin. Repeated administration of the inciting agents leads to chronic inflammatory changes.

In addition, activation of various components of the HF pathways, e.g., HF, prekallikrein, factor XI, plasminogen by cellular enzymes will be sought. In particular, alveolar macrophages from normal rabbits and rabbits with pulmonary inflammation will be tested. Aside from assaying the enzymatic activity of the components, critical cleavage of the protein structure will be examined. Previous studies in this laboratory have indicated that ^{125}I-labelled precursor components are activated by limited proteolysis.

Finally, as written in detail in the previous year's renewal, the crystals found in alveolar macrophages of smoker's lungs will be obtained and tested for their ability to activate HF. These fine crystals have the chemical composition and physical appearance of kaolin, an excellent activator of HF. The crystals are released periodically from the macrophages at which time serious inflammatory injury could take place if HF were bound and activated. These pilot studies, if positive, would lead to a series of studies aimed at finding the importance of such an activation mechanism inflammatory injury of the lung.

B. Cellular mechanisms involved in inflammatory tissue injury

These studies are designed to examine the mechanisms of activation of inflammatory cells and of release of their injurious constituents. Four cell types have been chosen for study; platelets, mast cells, macrophages and neutrophils. A series of activators for each cell type have been selected: for platelets - collagen, $F(Ab)_2$ anti-platelet antibody, platelet-activating factor derived from basophils (13) or pulmonary mast cells (14) and thrombin; for mast cells - polymyxin B, 48/80, C3a and C5a anaphylatoxins and a newly described cobra venom protein that stimulates mast cells to release granular constituents (11); for macrophages - immune complexes, components of the HF pathways, insolubilized aggregated immunoglobulins of specific classes and subclasses; for neutrophils-insolubilized aggregated immunoglobulins of specific classes and subclasses and C3a and C5a anaphylatoxins.

The studies will include examination of binding of the activators to the cell surfaces, the numbers of molecules bound that are required for release of constituents to occur and a definition of the receptors on the outer membranes of the cells with which the activators react.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Studies conducted in this laboratory have already demonstrated that several of these activators in insoluble form are capable of activating fully the respective cells to release their granular or lysosomal constituents (9). Different receptors apparently exist on mast cells for different stimuli and on neutrophils and platelets for different classes of immunoglobulins (8,11,12).

The mechanisms by which these activators stimulate release will also be examined. The suggestion that different esterase enzymes are activated by each stimulating agent will be tested experimentally (see below).

These experimental plans are presented only briefly as they represent a condensation of material given in detail in the initial application.

Experimental Plans for the Coming Year

A. Participation of the Hageman factor activated pathways in the pathogenesis of inflammatory tissue injury

1. The consumption of circulating ^{125}I prekallikrein, plasminogen, fibrinogen and clotting factor XI will be determined in rabbits given 200 µg purified lipopolysaccharide (LPS) of E. coli 0111:B4 and of the mutant Re 595 LPS. The latter possesses only the 2-keto-3 octanoic acid linkage group and lipid A and is an avid activator of the alternative pathway of complement. The former LPS fails to activate complement detectably. Both are potent activators of HF. Both forms of LPS when injected intravenously induce an inflammatory reaction with deposition of fibrin in the lung. The specific consumption of the above components will be assessed by comparing rates of loss from the circulation of the particular component in comparison to the loss of rabbit albumin.

Uptake of ^{125}I labelled components in the lung and other organs will be determined. Histologic and immunofluorescent studies will reveal the type of inflammation and the location of components of the HF and complement pathways in relation to the presence of inflammatory foci. Particular attention will be paid to the presence of neutrophils, platelets and macrophages. Animals used in these immunopathologic studies will be sacrificied at intervals of 30 min, 1, 4, 8 and 18 hours after a single injection of LPS. Damage to pulmonary alveolar walls will be assessed by the rate of passage of ^{131}I rabbit albumin into alveolar fluid obtained then by lavage in rabbits sacrificed sequentially. Lavage fluid will also be used to test for ^{125}I fibrinogen split products.

Similar analyses will then be conducted in rabbits using as inciting agents: (1) antibodies from sheep directed to rabbit pulmonary basement membranes; and (2) aerosolized finely particulate kaolin (an excellent activator of the HF pathways) coated with bovine albumin in rabbits previously sensitized with the antigen.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

In studies to be performed in the following year we will assess the affect of multiple administrations of these agents. Chronic changes, especially the development of fibrosis will be sought.

2. Depriviation studies to demonstrate the role of various factors in the development of inflammation.

In the currently proposed studies we will then deplete rabbits of cellular and humoral components to assess the effect on the development of inflammation and on the consumption and localization of other components as outlined above. The following studies will be performed initially:

a. Deprivation of neutrophils (by nitrogen mustard and specific anti-neutrophil antibody) and platelets (specific anti-platelet antibody).

b. Depletion of fibrinogen with commercially available snake venom enzyme (a technique we currently employ).

c. Blockade of the intrinsic clotting system by removing factor XI and factor VIII (trace proteins) with specific antibody. This method has proved successful in this and other laboratories.

d. Blockade of the extrinsic clotting system with antibodies to clotting factor VII. We are currently testing whether antibodies to bovine factor VII cross react sufficiently with rabbit factor VII to deplete this trace protein from the circulation. If the method does not succeed, we will purify rabbit factor VII to produce antibodies.

e. Removal of the third component of complement (C3) with cobra venom factor.

These deprivation studies are aimed at defining not only which components are important in the development of injury, but in what order they interact. A sequential activation sequence may then be recognized in the development of critical pulmonary injury.

3. The activation of components of the HF pathways by cellular constituents

Alveolar macrophages will be isolated from lungs of rabbits by lavage (Brain et al. Arch. of Int. Med. (26:477, 1970), a technique already employed in this laboratory, and the granules obtained by disruption of the cells in hypertonic sucrose. After lysis of the granules, the constituents will be assayed for their capacity to activate Hageman factor over a range of conditions of pH, temperature and ionic strength. Since Hageman factor, activated by enzymes such as plasmin or kallikrein is cleaved in the process, a similar effect of the granular constituents of alveolar macrophages will be examined. This will define the mechanism of activation as fluid phase rather than solid phase (i.e., when Hageman factor binds to negatively charged surfaces such as that provided by alveolar membranes). The implication would be that clotting of plasma, and release of kinins and plasmin would occur in the fluid of the alveolar spaces, thereby provoking a diffuse reaction.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

The granular constituents of the alveolar macrophages will then be fractionated on DEAE Sephadex A50 under conditions of constant pH and varying salt concentration. Enzymes or other activators of Hageman factor will be measured and isolated and identified through additional chromatographic and physical methods.

B. Mechanism of activation of cells important in the development of inflammatory injury.

We have observed that neutrophils, platelets, and mast cells release injurious products following stimulation at the membrane surface. In addition, considerable specificity exists in what might be considered as a membrane "receptor". We will now begin experiments in which the membrane receptors will be isolated and characterized. The work will initially be performed on neutrophils since large numbers of the cells (10^{10}) can readily be obtained in pure populations and since those cells play a fundamental role in pulmonary tissue injury.

Membrane fragments will be obtained from isolated washed neutrophils by shear-breakage in 0.34 M sucrose followed by sucrose density gradient centrifugation. Plasma membranes are found (40-60% recovery) at the interface between 20 and 40% sucrose. Granule breakage is minimal. We will use 5' nucleotidase and ^{125}I-labelled antineutrophil antibody (incubated with an aliquot of intact neutrophils beforehand) to follow the membranes.

The membrane fragments will be radiolabelled with iodine (lactoperoxidase-H_2O_2 method) and examined for binding to EAC 3_b (C 3b receptor) or Sepharose aggregated γ globulin (γG receptor). Preliminary experiments indicate the feasibility of the approach and the ability to obtain fragments retaining the C3b receptor. A binding inhibition assay will be established using unlabelled membrane to inhibit the binding of ^{125}I membrane to EAC or Sepharose γG. This will allow quantitative assessment of recovery of receptor from solubilized membranes. Four methods of solubilization will be examined: a) detergents, e.g., NP40 and Triton X100. The latter appears useful for the C3b receptor and may be removed afterwards by the method of Holloway (Holloway, P.W., Analytical Chem., 53, 309, 1973). b) Ultrasound. Low frequency ultrasonication of the fragments (to avoid oxidative effects) will be assessed. c) Proteolytic enzymes. Trypsin removes the C3b receptor from intact neutrophils. While trypsin probably fragments the receptor protein, the binding portion of this molecule may still be obtained and indeed preliminary evidence suggests that receptor activity can be recovered. d) Chaotropic agents and KCl. The different agents and techniques will be applied to the membrane fragment. The solubilization will be assessed by sucrose density gradient ultracentrifugation, examining protein and radiolabel and the receptor will be followed by binding inhibition.

Following solubilization, the receptors will be isolated by several means:

1. Affinity chromatography (see below in the mast cell section) using Sepharose-γG or zymosan particles with C3b attached (ZC) will entail binding of receptors to such particles followed by elution of the receptor molecules. For example, rabbit C3b receptors require Mg++ (Henson, Immunology, 1969) and thus may be eluted with EDTA.

2. Standard chromatographic procedures (with which this laboratory has much experience) will also be used to isolate the receptor molecules. These methods will include

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gel exclusion, ion exchange and hydroxyappatite chromatography, polyacrylamide gel electrophoresis, isoelectric focusing and pevikon electrophoresis. The receptors will thus be isolated and simultaneously characterized as to molecular weight, charge and composition.

3. The C3b receptor is presumably a protein (from its trypsin sensitivity) and is possibly a glycoprotein. Thus another approach to its isolation is to determine whether lectins e.g., concanavalin A or phytohemagglutinin, will bind to the receptor protein. If C3b binds to Sepharose-Con A, then this receptor may be eluted from the Sepharose-lectin with a-methyl mannoside.

Once isolated, the receptor will be labelled (e.g., with iodine if it is a protein) and studied for its binding properties and biologic activity. To indicate that it indeed may be the molecule involved in the cell activation, activation inhibition experiments, where the receptor is used to inhibit ZC induced phagocytosis on enzyme release, will be performed. Antibody to the receptor will be prepared and Fab fragments isolated. This antibody will then also be used to block neutrophil activation. The binding of radiolabelled receptor to EAC_{3b} or ZC_{3b} will be studied. What is the ratio of receptor to C3b in the erythrocyte? What is the nature of the bond, i.e., is it inhibited by salt or by detergents, indicating ionic or hydrophobic interactions (see section 1).

A question of considerable interest and importance which may then be answerable is whether C3b and C3a, both activators of neutrophils, may bind to and activate the same receptor.

C3b receptors will be studied for their ability to bind and remove active C3a. C3a will be assayed by anaphylatoxin activity or by neutrophil chemotaxis. These studies will allow us to follow C3b binding molecules during purification.

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12. Biographical sketches of investigator:

Charles G. Cochrane:

REDACTED

Education: University of Rochester, B.A.; M.D., School of Medicine

Professional Record: Department of Microbiological Chemistry (Prof. P. Grabar), Institut Pasteur, Paris, 1959-60; Assistant Professor, University of Pittsburgh, School of Medicine, 1960-61; Associate, Division of Experimental Pathology, Scripps Clinic and Research Foundation, 1961-64; Associate Member, 1964-67; Member, 1967-present; Professor of Pathology in Residence, University of California School of Medicine, 1968-present

Fellowships and Honors: Student Fellowship, Department of Pathology, University of Rochester, 1953-54; Sarah Mellon Scaife Fellow, Department of Pathology, University of Pittsburgh, School of Medicine, 1956-60; Helen Hay Whitney Research Fellow, 1961-64; Established Investigator, The Helen Hay Whitney Foundation, 1964-69; John M. Sheldon Memorial Lectureship of the American Academy of Allergy, 1968; Parke, Davis Meritorious Award in Experimental Pathology for the Year, 1969.

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Consultantships: Member, Pathology Training Committee, National Institute of General Medical Sciences, USPHS; Fellowship Committee, The Arthritis Foundation; Member of the Advisory Committee, National Multiple Sclerosis Society.

Editorial Boards: The Journal of Clinical and Experimental Immunology, The American Journal of Pathology, Associate Editor, The Journal of Immunology.

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12.

12. Biographical sketeches of investigators:

Peter M. Henson:

REDACTED

Education: High School in Britain and Rhodesia

Bachelor of Veterinary Medicine and Surgery (BVM&S), Edinburgh University, Edinburgh, Scotland, R William Dick Medal for best graduate.

First Class Honors, B. Sc. in Bacteriology, Edinburgh University, Edinburgh, Scotland, R

Ph. D., University of Cambridge, Cambridge, England, R Thesis: "The Biological Activity of the Third Component of Complement".

Professional Record:

Research Fellow, Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, CA 92037

Associate, Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, CA 92037

Associate Member, Department of Immunopathology (same as above), Scripps Clinic and Research Foundation, La Jolla, CA 92037

Memberships:

REDACTED

REDACTED

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12. Biographical sketches of investigators:

Stephen W. Russell

REDACTED

Education:

University of California, Davis, REDACTED B. S.

University of California, Davis, REDACTED D.V.M.

Internship, Angell Memorial Animal Hospital, Boston, Massachusetts, REDACTED

University of California, Davis, REDACTED and University of California, San Francisco, REDACTED Ph. D.

Professional Record:

Postdoctoral Research Fellow, Scripps Clinic and Research Foundation, La Jolla, CA 1972-1973

Associate, Scripps Clinic and Research Foundation, La Jolla, CA 1973-present

Awards and Academic Honors:

Special Fellow, United States Publich Health Service, 1970 & 1971
Outstanding Clinical Research Project, Angell Memorial Animal Hospital, 1967
University of California School Medalist, 1966
Graduated 1st in Veterinary Medical School Class, 1966
Outstanding Clinician, Veterinary Medical School Class, 1966
Hart Memorial Scholarships, 1964 and 1965
B. S. Degree with Honors

Professional Organizations:

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REDACTED REDACTED

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12. Biographical sketches of investigators:

Richard J. Ulevitch, Ph. D.

REDACTED

Education:

A.B., Liberal Arts, Washington and Jefferson College, R

Ph. D., Biochemistry, University of Pennsylvania, R

Professional Record:

1971 - 1972, Postdoctoral fellow, University of Minnesota with Drs. Beulah Holmes Gray and Robert A. Good

1972-1974, Research Fellow, Scripps Clinic and Research Foundation, Department of Immunopathology, La Jolla, CA 92037

1974-present, Research Associate, Department of Immunopathology, Scripps Clinic and Research Foundation

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Progress Report

1. Participation of the intrinsic clotting, kinin forming and fibrinolytic systems in disease

a. Biologic activities of the first component, Hageman factor (HF)

Purified, radiolabelled HF has been examined as to its physical properties and biologic activities. These studies are of significance in that when HF is activated, it binds (solid phase activation) to the negatively charged activator, leading to generation of numerous potentially injurious agents: kinins, fibrinogen split products, fibrin; activated C1s, neutrophilic chemotactic factors and active esteratic enzymes.

The amino acid composition of HF was determined (1). This will be of help in future studies of the residues important in the biologic activities of HF. Antibodies were produced to human HF and quantitative determinations of the molecule in plasma or other fluids is possible for the first time. Normal values range from 14 to 47 µg/ml plasma with an average value of 29 µg/ml. Studies on the levels of this important protein in disease are underway.

As noted in last year's report, HF is cleaved by enzymes that it activates which, in a reciprocal fashion, activate additional HF. We have termed this "fluid-phase" or enzymatic activation (2,3, 4). The HF molecule is cleaved into fragments of 52,000, 40,000 and 28,000 daltons. The 28,000 MW fragment contains the enzyme sites of the molecule (1). We have recently observed that the sites responsible for binding the HF to negatively charged surfaces (solid-phase activation) lie in the 52,000 and 40,000 MW fragments. When HF, bound to and activated by a negatively charged surface (such as provided by collagen or extracellular basement membranes of the lung) is exposed to its substrates in plasma, namely prekallikrein and plasminogen. These enzymes cleave fresh, native HF and activate it. They also cleave the bound HF, and release the 28,000 enzymatically active fragment into the supernatant (2, 4). The active fragment then becomes rapidly bound to an inhibitor which is most probably C1 inhibitor: the fragment does not bind to the inhibitor in C1 inh. deficient plasma; the fragment binds to purified C1 inh. and the 28,000 MW fragment changes its electrophoretic mobility in whole plasma to become superimposable with the C1 inh.

From these studies, one may anticipate that HF when activated in diseases will be found along membranes where it is bound. The portion bound will be the 52,000 and 40,000 M.W. fragments only, while the 28,000 M.W. fragment will be present in the plasma, bound to C1 inhibitor. We are currently designing assays for the activated 28,000 M.W. fragment in plasma. It is our feeling that far more sensitive assays can be obtained for the activated HF than for the residual native molecules. The same theory applies to kallikrein, factor XI and plasmin.

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b. Purification of rabbit plasminogen, fibrinogen, clotting factor XI and prekallikrein for assays of consumption during inflammatory injury.

In order to examine the participation of these proteins in inflammatory pulmonary injury, it is essential to obtain them in purified, precursor form. Radiolabels can then be applied and antibodies obtained for detection in plasma, alveolar fluid and tissues. Accordingly, we have obtained each of these in precursor form by dermatographic methods. While inactive as they exist, each can be converted to its active form by treatment with its natural activator, i.e., plasminogen activator, thrombin, and HF respectively. We are now obtaining turn over times of the radiolabelled molecules in normal rabbits prior to performing studies of turn over (consumption) and tissue-localization in inflammatory conditions of the lung (see original report). Antibodies are now prepared to fibrinogen and plasminogen for quantitative assays, and goats are being immunized with prekallikrein and factor XI.

c. Action of alveolar macrophages on components of the HF pathways.

The initial studies of the activation of prekallikrein and HF by alveolar macrophages have been made difficult by the low concentrations of the components in solution. We have therefore expended considerable effort in developing a new method for preparing components of the HF pathway that will yield much greater quantities. The method, as reviewed in the section on experimental protocols for the ensuing year, involves a computer programmed method of preparatory polyacrylamide electrophoresis (Chrombach and Rodbard). We have established the computer programs which describe approximately 5,000 buffer conditions available for isolating any given protein by its physical properties. We have applied the technique to rabbit prekallikrein with extraordinary results: far greater amounts of the prekallikrein were obtained than before in purer form. When the proteins of the HF pathway are prepared in adequate concentration by the new method, we will continue the studies that appear most promising: the activation of components of the HF pathways by enzymes obtained from alveolar macrophages.

d. Activation of Hageman factor by bacterial lipopolysaccharide.

Hageman factor was formed to be activated by purified bacterial lipopolysaccharide (LPS) (5,6). The lipid A moiety was determined to be the active portion in the LPS structure. Hageman factor bound to the LPS and was active in the complexed form. Cleavage of the molecule did not occur.

e. The identification of PF/dil.

When plasma is diluted with saline in the presence of a negatively charged surface, a permeability factor (PF/dil) is generated. The nature of this substance has remained unknown since its discovery in 1953, although its interaction with the Hageman factor pathway appeared certain. Using insolubilized antibodies to Hageman factor and other components of the pathway, we determined that PF/dil was identical with activated Hageman factor (7,15).

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2. Mechanism of activation of neutrophils, platelets and mast cells for release of injurious constituents.

Studies were continued into the mechanisms of activation of inflammatory cells and release of their injurious constituents. These studies were designed to mimic conditions occuring in inflammatory foci where neutrophils, platelets, macrophages, mast cells and lymphocytes release constituents that injure surrounding tissues. The stimulation of platelet surfaces was brought about by several agents: $F(Ab)_2$ anti-platelet antibody, platelet activating factor (PAF) derived from basophils and pulmonary macrophages, collagen and thrombin. Each agent induced release of stored 3H serotonin (8). To determine whether these chemically disparate agents activated release mechanisms in the platelet by different means, the characteristics of the esterase enzymes which are activated early in the release process were examined. Using phosphonate inhibitors, synthetic ester substrates, and plant-derived inhibitors of trypsin, it was found that each activator activated esterases in the platelets possessing different inhibitory spectra. This strongly suggested different esterases are involved and that specific receptors are activated by each of the above stimuli.

With mast cells derived from peritoneal cavities of rats, two types of activators were described that induce release of histamine and serotonin. Type I activators included compound 40/80 and polymyxin B while Type II activators included C3a anaphylatoxin, a cobra venom protein recently described in this laboratory, and band 2 protein of neutrophils (9, 16). These activators were found to stimulate the mast cells at their surface (10), for insolubilization of the activators on Sepharose 4B allowed full activation to occur. The differentiation into two types of activators was determined by blocking the binding of one type of activator (radiolabelled with ^{125}I) with members of that type, but not the other, and by the ability of activators of one type to desensitize mast cells to activators of that type, but not the other (11, 12).

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R: REDACTED MATERIAL

THOMAS CLARKE KRAVIS, M. D.

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Education:

Fairfield University
Fairfield, Connecticut
B.S. Biology - cum laude - R
Activities and honors: Dean's list

Jefferson Medical College
Philadelphia, Pennsylvania
M. D. - R
Activities and honors: Dean's committee; student council; vice president; director: Project Haiti; president: Student AMA

University of California, San Diego
Straight medical internship - Dr. E. Braunwald, 1968-1969
Medical resident - Dr. E. Braunwald, 1969-1970
Pulmonary clinical fellow - Dr. K. Moser, 1970-1971
Pulmonary research fellow - Dr. N. Zvaifler, 1971-1972
Research fellow - Dr. C. Cochrane - Scripps Clinic & Research Foundation, 1972-
Clinical instructor of medicine - U. C. S. D., 1972-

Activities:

Director medical clinic teaching program - Tijuana Mexico (1969); Instructor student medical clinic (1971); Coordinator pulmonary rehabilitation course (1971); lectures in inhalation therapy, Grossmon College (1970); Director alpha-1-anti-trypsin laboratory, U. C. S. D. and coordinator alpha-1-antitrypsin SCOR study.

Current studies:

The role of IgE and the basophil in immune complex disease; platelet activating factor in asthma; leukocyte dependent histamine release in man and rabbit. Antigen-antibody crossed gel electrophoresis for alpha-1-antitrypsin phenotypes.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Approximately 2,000 sq. ft. of laboratory space is available. The laboratory is equipped with refrigerated centrifuges, electrophoretic and chromatographic equipment, five fraction collectors, a walk-in cold room, deep freezes (-20° and -70°), complete fluorescent equipment and a preparative ultracentrifuge. Histologic and electron microscopic analyses are carried out in facilities available and frequently used by the applicants. An analytical ultracentrifuge and amino acid analyzer are also used by the applicants within the department. Complete isotope labeling, hot lab and monitoring facilities are in current use. Two Schultz-Dale bath apparatuses are used for assays of biologically active materials.

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

Charles G. Cochrane, M.D.; Peter M. Henson, Ph.D.; Stephen W. Russell, DVM, Ph.D.; Richard J. Ulevitch, Ph.D.; Thomas C. Kravis, M.D.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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4.

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Charles G. Cochrane	20	
Peter M. Henson	20	
Stephen W. Russell	50	
Richard J. Ulevitch	50	
Fringe Benefits – S. W. Russell R. J. Ulevitch		
Thomas C. Kravis Technical	50	
Part time services of animal caretaker and dishwasher		
Fringe Benefits		
	Sub-Total for A	
B. Consumable supplies (by major categories)		
Chemicals, proteins		1,500
Glassware, plasticware		1,350
Animals, feed and bedding		1,000
	Sub-Total for B	3,850
C. Other expenses (itemize)		
none		---
	Sub-Total for C	---
	Running Total of A + B + C	22,450
D. Permanent equipment (itemize)		
none		
	Sub-Total for D	
E. Indirect costs (15% of A+B+C)	E	3,368
	Total request	25,818

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Immunologic Studies	United States Public Health Service AI07007	$150,000	1975-1976
The Mediation of Inflammatory Injury of Tissues	Council for Tobaccco Res.	23,400	1975-1976

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Charles G. Cochrane, M. D.

Signature [signature] Date Jan. 28, 1975

Telephone 714 (Area Code) 459-2390 (Number) 306 (Extension)

Checks payable to

Robert Erra

Mailing address for checks

Scripps Clinic and Research Foundation

476 Prospect Street, La Jolla, CA 92037

Responsible officer of institution

Typed Name Jacinto J. Vazquez, M. D.

Title Assistant Director

Signature [signature] Date Jan. 28, 1975

Telephone 714 (Area Code) 459-2390 (Number) 223 (Extension)

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References

1. Revak, S., Cochrane, C., Johnston, A., and Hugli,T.: Structural changes accompanying enzymatic activation of human Hageman factor, J. Clin. Invest., 54: 1974.

2. Cochrane, C.G., Revak, S.D., and Wuepper, K.D.: Activation of Hageman factor in solid and fluid phases, J. Exp. Med. 138: 1973.

3. Cochrane, C.G., Revak, S.D., Wuepper, KD., Johnston, A., Morrison, D.C., and Ulevitch, R. :Activation of Hageman factor and the kinin forming, intrinsic clotting, and fibrinolytic systems, Adv. in the Biosciences, 12:1973.

4. Cochrane, C., Revak, S., Wuepper, K., Johnston, A., Morrison, D., and Ulevitch, R. : Soluble medicators of injury of the microvasculature Hageman factor and the kinin forming, intrinsic clotting and the fibrinolytic systems, Microvascular Research 8: 112, 1974.

5. Morrison, D.C., and Cochrane, C.G.: Direct evidence for HF (factor XII) activation by bacterial LPS (endotoxins), J. Exp. Med. 140: 797, 1974.

6. Cochrane, C.G.: Hageman factor: its characterization and potential participation in inflammation, International Symposium on Inflammation, 5-3-74, in press.

7. Johnston, A., Cochrane, C., Revak, S. The relationship between PF/dil and activated human Hageman factor, J. Immunol. 113:103, 1974.

8. Henson, P.M., Fed. Proc. 33: 797, 1974.

9. Morrison, D.C., Roser, J.F., Henson, P.M., and Cochrane, C.G. Activation of rat mast cells by low molecular weight stimuli, J. of Immunol. 112: 573: 1974.

10. Morrison, D.C., Roser, J., Cochrane, C.G., Henson, P.M.: Surface membrane stimulation induces mast cell degranulation, accepted J. of Immunol. 1975.

11. Morrison, D.C., Henson, P.M., Cochrane, C.G.: The activation of inflammatory cells in immunologic disease, submitted to Intnl. Congr. of Rheum., London, Spring 1974.

12. Cochrane, C.G.:The participation of cells in the inflammatory injury of tissue, sub. J. Invest. Derm., 12/74.

13. Benveniste, J., Henson, P.M., Cochrane, C.G. Leukocyte-dependent histamine release from rabbit platelets, J. Exp. Med. 136: 1356-1377, December 1972.

14. Kravis, T., and Henson, P.M., Fed. Proc. 33: 797, 1974.

15. Ulevitch, R.J., Cochrane, C.G., Revak, S.D., Morrison, D.C., and Johnston, A. :The structural and enzymatic properties of the components of the Hageman factor activated pathways, sub. Cold Spring Harbor Symp., 10/1/74

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16. Cochrane, C.G., Janoff, A., The Arthus Reaction, Inflammatory Proc.,III: 85, 1974.

17. Russell, S.W., Francke, U., Buettner, L., and Cochrane, C.G.:Modes of growth and spread of a transplantable, virus-producing murine (Moloney) sarcoma: karyotypic analyses. J. Nat'l. Cancer Inst., 53: 801, 1974.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

January 31, 1975

Grant Application No. 954A

PULMONARY

To: The committee comprising Drs. Gardner, Liebow and Sommers

Subject: Allen B. Cohen, M.D., Temple University, Philadelphia
Continuation application No. 954A
"The Genetic Defect in Alpha-1-antitrypsin Deficient Patients"

History

Dr. Cohen, originally located at the University of California Service, San Francisco, has been supported by CTR since April 1971. The project, (CTR Grant No. 808) on "Human Alveolar Macrophages and Emphysema" terminated March 31, 1974.

At the March 1974 SAB meeting, a new grant,#954, was approved for a three year program in the amount of $41,000 for the first year (May 1, 1974 through April 30, 1975), to support studies entitled "The Genetic Defect in Alpha-1-antitrypsin Deficient Patients".

As of July 1, 1975 Dr. Cohen will move to Temple University, Pa. and a supplementary grant application requesting $5,326 has been submitted to the CTR to cover the period May 1, 1975 through June 30, 1975: this will be handled administratively.

The emphasis of the future program will be changed from defining the genetic defect in AAT-deficient patients, to determining the mechanism of the active site of AAT (see letter enclosed).

To allow full discussion of this proposal, the application has been treated as a Continuation Proposal (#954A) rather than as a first-year renewal application, and so will be placed in the SAB agenda for the March 1975 meeting.

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Allen B. Cohen January 31, 1975

Request

Grant application No. 954A requests $65,762 for the first year of a two year program.

Documents submitted (attached)

1. Application dated January 20, 1975 (9 pages including C.V. of Dr. Turner).
2. Progress Report (13 pages including Tables and Diagrams).
3. Two abstracts (1 page each).
4. One letter (2 pages).

D.S.

DS:wg
Encl.

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454A

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8865

JAN 8 0 1975

Application For Renewal of Research Graht

(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐ Date: 1/20/75

1. Principal Investigator (give title and degrees):

Allen B. Cohen, M.D., Ph.D., Associate Professor of Medicine

2. Institution & address:

Temple University
Health Sciences Center
3420 North Broad Street
Philadelphia, Pennsylvania 19140

3. Department(s) where research will be done or collaboration provided:

Department of Medicine

4. Short title of study:

The Genetic Defect in Alpha-1-antitrypsin Deficient Patients

5. Proposed renewal date: July 1, 1975-Grant for year 2

6. How results to date have changed earlier specific research aims:

See appended Sheets

7. How results to date have changed earlier working hypothesis:

Results to date have not changed the earlier working hypothesis.

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8. Any additional facilities now required? Describe briefly:

New facilities at Temple University include about 1,000 sq. ft. of newly renovated laboratory space. This space represents about a 30% increase over my existing facilities at University of California. All of my equipment can be moved with me to Temple University and no additional facilities will be required.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Mrs. Dagmar Geczy, my chief laboratory technician will go with me to Temple University. This should minimize the transition time.

Dr. Lo will not move to Temple U. In her place, Dr. Richard Turner will join me at Temple. Dr. Turner did his Ph.D. at the University of Minn. and a post-doctoral fellowship at this institution with Dr. Irving Liener, a well known research scientist in the area of protein inhibitors of proteolytic enzymes in plant systems. Therefore, Dr. Turner is admirably suited to continue the work on AAT. Bibliography is appended.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

Abstracts:

Lo, T.W. and Cohen, A.B.: The Interaction between alpha-1-antitrypsin and sepharose-bound elastase. In Press. Fed. Proc. Accepted for presentation at the American Society of Biological Chemists, national meeting, Atlantic City, May 15, 1975.

Cohen, A.B.: A Biochemical Approach to Human Emphysema. Submitted to the American Lung Association for consideration for presentation at their annual meeting in Montreal, May 18, 1975. Will be publised in the American Review of Respiratory Diseases if accepted for publication.

REVIEW: Cohen, A.B. Ch. 8. Alpha-1-antitrypsin: A systemic Determinant of Lung Structure and Function. In the Biochemical Basis of Pulmonary Function, Ed. R. Crystal. A volume in the series: "Lung Biology in Health and Disease", Ed.C. Lenfant. Marcell Dekker, Inc., New York, In Press.

12. Summary progress report (append in standard form as separate document, unless recently submitted).

See page

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13. Budget for the coming year: 7/1/ 75-6/30/76

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Allen B. Cohen	30%	
Richard Turner (Base $12,000)	100%	REDACTED
Technical		
Mrs. Dagman Geczy (Base $16,100)	100%	REDACTED
(Salaries include 15% fringe benefits)		
		REDACTED
Sub-Total for A		

B. Consumable supplies (by major categories)

Enzymes	$2050	Ampholytes (25 runs)	$ 550
Substrates	$1550	General Chemicals	$1585
Column resins	$ 850	Pipettes (Vol. & Micro)	$ 550
Glassware	$1050	Isotopes	$ 750
Miscellaneous laboratory and office supplies			$1250

Sub-Total for B $10,185

C. Other expenses (itemize)

Animals for immunization	$3445
Blood for alpha-1-antitrypsin preps (200 units)	$5145
Publication costs (reprints and page charges)	$1645
Service contracts on equipment	$2145
Travel ($500 for each professional)	$1000

Sub-Total for C $13,380

Running Total of A + B + C $55,880

D: Permanent equipment (itemize)

Miscellaneous items of equipment. This will probably include another power supply and another polyacrylamide disc gel electrophoresis apparatus since our current equipment is overutilized.

Sub-Total for D $ 1,500

E. Indirect costs (15% of A+B+C) E $ 8,382

Total request $65,762

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13. Budget for the coming year:

BUDGET COMMENTARY

For First Renewal - July, 1975

1. As shown on page 4 of the application, a grant request submitted to the National Heart and Lung Institute is pending. The grant is similar to this application but would start in September, 1975. If both grants are funded to the extent requested, the 1976 renewal of the Tobacco Research Council grant will not be requested, and the balance of this first renewal following the starting date of the NHLI grant will be returned to the Council.

Application for a supplemental grant from the Council for Tobacco Research for two months (May-June, 1975) for $5,326 has been requested through the University of California,San Francisco, at the request of Dr. Robert Hockett, and this request is pending.

2. The salary for the Ph.D. position (Dr.Richard Turner) was budgeted on the original grant request.

3. Items in budget categories B and C have been computed by adding 5% to the request for the first year.

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R: REDACTED MATERIAL

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None after July 1, 1975			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
A Biochemical Approach to Human Emphysema	National Heart and Lung Institute	140,575 (year 1)	September, 1975-1980
(See budget Commentary, item #1 regarding this grant)			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name: Allen B. Cohen, M.D., PH.D.

Signature: [signature] Date: 1-7-75

Telephone: (415) 648-8200 ext. 273 or 627
Area Code Number Extension

Checks payable to

Temple University

Mailing address for check:

Mr. David W. Siegel, Assoc. Vice Pres. Fin.
Temple Univ. Health Sciences Center
~~3120 North Broad St., Philadelphia, Pa. 19140~~

Responsible officer of institution:

Typed Name: Dr. Samuel S. Herman

Title: Assoc. Vice President, Research Administration

Signature: [signature] Date: 1/25/75

Telephone: REDACTED
Area Code Extension

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

6. How results to date have changed earlier specific research aims:

Our original plan required the isolation of alpha-1-antitrypsin from patients with a genetically determined deficiency of AAT in their blood. Since this abnormal protein occurs in low concentrations and is very unstable in patients with this disease, conventional means of isolation of the protein have not been successful. One of the approaches which we have employed to isolate the abnormal AAT is based on the knowledge that AAT binds to various proteolytic enzymes. There are only two elastase inhibitors in human serum. One of them is AAT and the other is more than 20 times heavier than AAT. Therefore, an elastase affinity column was thought to be a logical approach to the isolation of AAT. The details of the experiments are presented in the progress report. In brief, the AAT which was washed from the elastase column was no longer an enzyme inhibitor and it had lost a peptide of about 4,000 daltons molecular weight. When the interaction between isolated AAT and elastase was then studied in vitro with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) it was evident that AAT exhibited similar behavior in solution. These results made it evident that while the elastase affinity column could not be used to isolate abnormal AAT for structural studies, the elastase on the column might have cleaved AAT at the inhibitory site and offered a convenient method for determining the amino acid residue at the inhibitory site. Since this information is the key to developing reagents which might replace AAT in patients with a deficiency of this serum protein, we would like to pursue the study of the elastase inhibitory site with the affinity column. In addition, the techniques developed during this study may permit us to identify the key amino acids at the other enzyme inhibitory sites on AAT. Other lines of investigation of the inhibitory sites of AAT in progress in our laboratory will permit us to confirm the data from the affinity column by other means. These experiments are alluded to in the progress report.

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9. Biographical sketch of new key personnel:

Curriculum Vitae

Personal: name Richard H. Turner

REDACTED

current address

REDACTED

office phone 1-612-3-3-1281

marital status REDACTED

Extra-professional interests:

foreign language and travel fluent in German and Spanish reading knowledge of French extensively travelled throughout Western Europe, Canada and Mexico

music proficiency in classical violin and fiddle concert performances with the Minneapolis and St. Paul Civic Orchestras

business economics portfolio manager and co-trustees of the James E. Turner Trust Fund director on the board of Engineering Products Co. of Waukesha, Wisconsin engaged in trading stocks and bonds on the New York Stock Exchange and options on the Chicago Board of Options

counselor for Project Motivation an organization dealing with disadvantaged children in the Twin Cities

sports intramural atheletics

Honors:

NIH trainee 1968-73

postdoctoral training under a grant from Proctor and Gamble 1974

recipient of the Wisconsin State German Teachers Competition The first prize was a six-week cultural exchange with German families in Bonn, Vienna, and Berlin.

Academic Training:

B.A. Carleton College 1968 major biology

Ph.D. University of Minnesota major biochemistry minor physical and organic chemistry

In addition to coursework in the major and minor fields, the traineeship provided the opportunity to teach at both the undergraduate and graduate levels. This included organizing and preparing laboratory exercises and a limited number of lectures.

Research interests: My thesis work involved the isolation and characterization of soybean proteins by classical methods. The plant protein proteinase inhibitors which were isolated were derivatized by a number of organic reactions. The interaction of the inhibitors with proteases was characterized with regard to their thermodynamics and kinetics. This aspect of the thesis will soon be submitted to Biochemistry for publication.

My current research is involved with the high speed isolation of plant lectins and detoxification of soy flour by chromatographic procedures. The purified lectins will be used to probe changes in malignant cell membrane structure.

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10. Plan for the Coming Year:

From the work in our laboratory during the past year, a hypothesis can be proposed for the biochemical mechanism of AAT and the hypothesis can be subjected to experimental tests. Earlier, we proposed that AAT interacted with enzymes in a manner similar to their interaction with protein substrates but instead of cleaving the substrate, AAT and the enzyme formed a stable intermediate. The interaction of serine proteases and substrates has been schematized by Stroud (Fig. 10-1). The schematic shows the different intermediates which develop during the interaction of serine proteases with substrates. Recently, Moroi (Biochem. Biophys. Acta. 359:130, 1974) has suggested that the AAT-enzyme complex is one of these intermediates, called the acyl intermediate (Panel 4, Fig. 10-1). In this intermediate the peptide on the amino-group side of the peptide cleavate has fallen from the complex and the serine hydroxyl at the enzyme catalytic site is bound to the carboxyl group of the amino acid at the end of the remaining protein fragment. This bond is an ester bond, and as mentioned in the progress report these bonds are often susceptible to cleavage under mild alkaline conditions. Our work in the past year has shown that the AAT in the AAT-enzyme complex has already lost a peptide and that the loss of the peptide is not dependent on unbound or active trypsin in the solution. In addition, the bond between the AAT and trypsin is broken under mild alkaline conditions. These data are highly suggestive that the AAT-enzyme complex is indeed an acyl intermediate (Fig. 10-2).

During the ensuing year we wish to test the next step in the hypothesis: Enzymes are attached by an alkaline labile bond to the new carboxy terminal amino acid on AAT. The design of the experiments are diagramed in Figure 10-3.

We have already determined that KOH can cleave the AAT-trypsin complex into an active enzyme and a fragment of AAT with a small peptide missing. If the enzyme is bound to the carboxy-terminal amino acid through its carboxyl group, as it must be to fulfill the requirements of the hypothesis, then the AAT in the complex will have no chemically recognizable carboxy-terminal amino acid. Carboxypeptidases are enzymes which cleave amino acids from the carboxy-terminal end of proteins, one residue at a time. Therefore, if the AAT-enzyme complex is an acyl intermediate and if a carboxypeptidase of appropriate specificity is added to the complex, the carboxy-terminal amino acid of the enzyme-bound fragment of AAT will not be removed (Fig. 10-3, left arrows). However, if the complex if first dissociated and then the carboxypeptidase is added, the amino acid at the enzyme inhibitory site will be removed and determined in the amino acid analyzer (Fig. 10-3 right arrows).

The experiment can be further illustrated by describing its use in determining the amino acid at the inhibitory site on AAT for trypsin. Carboxypeptidase B (CPB) is an enzyme which cleaves arginine and lysine amino acids from the carboxy-terminal end of proteins and trypsin cleaves proteins at arginine and lysine residues inside of the peptide chain. Therefore, a solution containing AAT-trypsin complexes will be divided into two aliquots and one aliquot will be dissociated with KOH. Then both aliquots will be treated with CPB. Amino acids which are released will be analyzed on an amino acid analyzer. If trypsin is bound to carboxy-terminal arginine or lysine on the remaining AAT peptide, then an arginine or lysine should be removed only from the complex dissociated in KOH. This experiment would have the dual function of demonstrating that trypsin is bound to the carboxy-terminal amino acid of the remaining peptide, and identifying this key amino acid. All enzymes inhibited by this

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site on AAT would have to be able to cleave proteins at this same amino acid; therefore, if an inhibitor were designed by starting with this amino acid and adding a binding group, then we could say with certainty that the resultant inhibitor had the same function as the AAT inhibitory site which it was designed to mimic.

Other inhibitory sites on AAT may be identifiable in the same manner.

Significance of the Research:

Individuals with a severe deficiency of AAT in plasma have a high incidence of emphysema. Since AAT is known to be an enzyme inhibitor, emphysema in such patients might logically be attributed to the unrestrained action of an enzyme or several enzymes on pulmonary tissues. A chemical substitute for the missing inhibitor is of obvious clinical importance. If the hypothesis for the biochemical mechanism of action of AAT presented in this protocal is correct, it may provide a basis for developing drugs that block enzymes inhibited by AAT and prevent the development of emphysema in deficient patients. In addition, if an excess of similar enzymes, not blocked by normal levels of inhibitor, is found to cause other types of emphysema, then the drugs may be useful in preventing emphysema in patients other than those with a deficiency of AAT.

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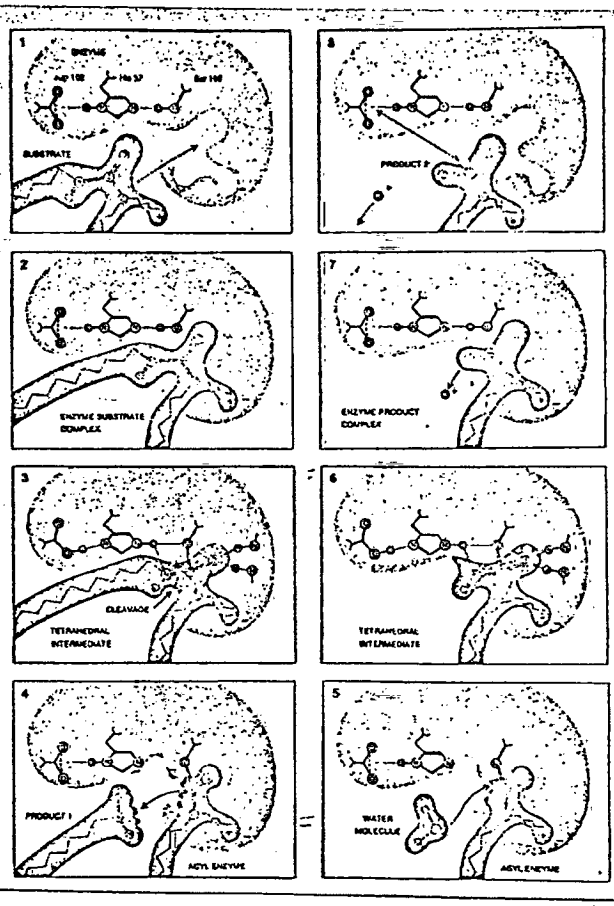

Figure 10-1:
CATALYTIC MECHANISM of trypsin and other serine proteases accelerates the hydrolysis of peptide bonds by providing intermediate states for the reaction and by smoothing the transition from one intermediate to the next. Enzyme and substrate must first come together (1) to form a precisely oriented complex (2). The oxygen atom of serine 195 then bonds covalently to the substrate carbon, forming a tetrahedral intermediate (3); the proton, or hydrogen ion, from serine 195 is transferred to the substrate nitrogen. The proton transfer breaks the peptide bond, and the first product is liberated (4). The remaining complex is called an acyl enzyme; it breaks down to regenerate free enzyme in steps that are symmetrical with those of the first half of the process. A water molecule enters the reaction (5), its hydroxyl group forming with the substrate another tetrahedral intermediate (6). The hydrogen ion from the water molecule is transferred to serine 195, breaking the covalent bond between enxyme and substrate (7). The second product is now freed (8); its departure is hastened by repulsion between negatively charged carboxyl groups of product and aspartic acid 102. Histidine 57 and aspartic acid 102 participate in the proton transfers.
(R.M.Stroud, Sci.Am., 237:74, 1974)

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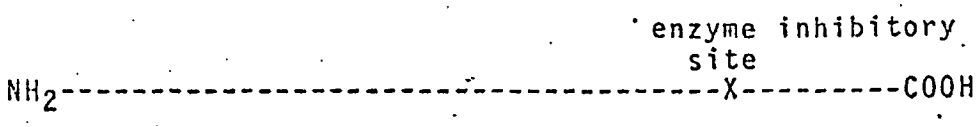
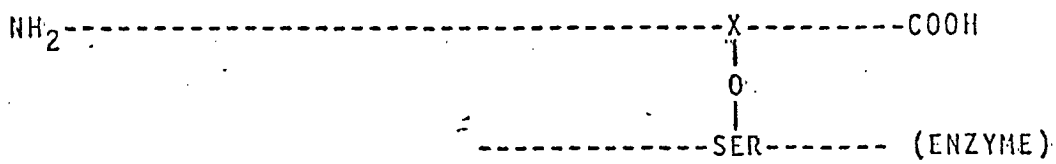
1. ALPHA-1-ANTITRYPSIN



2. ALPHA-1-ANTITRYPSIN-ENZYME COMPLEX

(Tetrahedral Intermediate)



3. ALPHA-1-ANTITRYPSIN-ENZYME COMPLEX

(Acyl Intermediate)

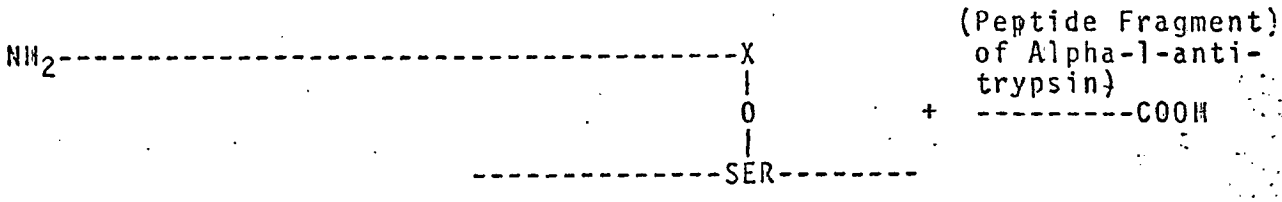


Figure. 10-2: Diagram of the Hypothesized Stages of Interaction of AAT with Enzymes.
SER= the serine amino acid at the catalytic site of the enzyme.
X = the amino acid at the inhibitory site of AAT.

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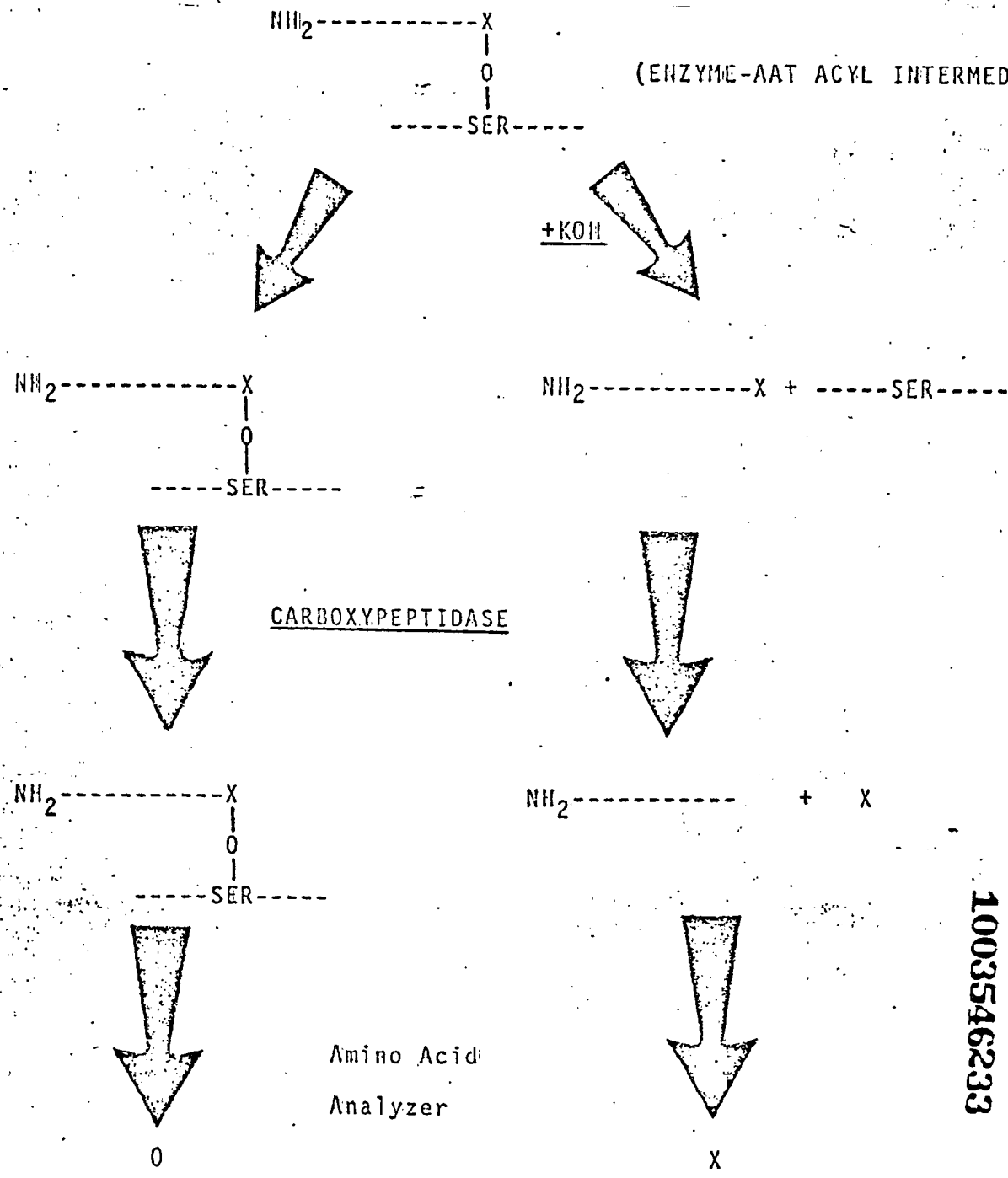

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Fig. 10-3: Diagram of Experiment Designed to Determine if Enzymes are Bound to AAT at the Carboxyl Terminal Amino Acid of AAT and if They are, to Determine the Identity of the Amino Acid (X) at The Inhibitory Site of AAT.

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CTR Grant #954

Progress Report #1

Allen B. Cohen, M.D., Ph.D.
University of California, San Francisco
C/O Chest Division
San Francisco General Hospital
San Francisco, California 94110

May 1, 1974 to
January 15, 1975

THE GENETIC DEFECT IN ALPHA-1-ANTITRYPSIN IN DEFICIENT PATIENTS

The Interaction Between Alpha-1-antitrypsin (AAT) and Elastase:

The rationale for our original study of the elastase affinity column is outlined under item 6 of the renewal application. Briefly, an elastase affinity column seemed to be a logical method for attempting to isolate AAT of ZZ phenotype. However, examination of the products which are washed from an elastase column when AAT is added to it revealed that the AAT had lost its enzyme inhibitory activity and had lost a peptide which comprised less than 10% of its molecular weight. These experiments are reviewed below. The results seem to offer an unique opportunity to study some of the products of the interaction between AAT and elastase and perhaps to identify the key amino acid at the elastase inhibitory site of AAT.

Methods and Results:

The reactions of AAT with both soluble and sepharose-bound elastase were examined. The concentrations of enzymes and inhibitor were determined employing the extinction coefficient of individual protein. The catalytic activities of trypsin and elastase in hydrolyzing p-Toluenesulfonyl-L-Arginine Methyl Ester (TAME) and N-Acetyl-L-alanyl-L-alanyl-Lalanyl Methyl Ester (AAME), respectively, were measured with a Radiometer pH Stat as described by Gertler and Hofmann (1). Trypsin activity was also analyzed by the method of Erlanger, et. al. (2), using N-Benzoyl-DL-Arginine-p-Nitroanilide (BAPNA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the procedure of Laemmle (3) as modified by Hokin and co-workers (4). Bovine elastase (76 mg) was coupled to Cyanogen Bromide-activated Sepharose-4B (50 ml) following directions given by Pharmacia Fine Chemicals. The Sepharose-bound elastase hydrolyzed AMEE. A column packed with Sepharose-bound elastase was equilibrated with 0.1M Tris, pH 7.6 containing 0.5M NaCl. Purified and active AAT (20 mg) in the same buffer was applied to the column and two protein containing peaks were washed from the column (Fig. 1). The protein in the first peak showed a precipitin band with antisera against AAT in immunodiffusion analysis. However, it no longer inhibited the activity of trypsin when assayed with TAME and BAPNA or of elastase when assayed with AAME. The molecular weights of active and inactive AAT were estimated to be 54,000 and 50,000 daltons, respectively, by SDS-PAGE (gels 1 and 2 of Fig. 2). In addition, the reduced and non-reduced inactive AAT had identical mobilities (gels 2 and 4 of Fig. 2). The protein in the second peak passed through a molecular sieving filter which retains molecules in excess of 10,000 daltons, and it showed a single band in SDS-PAGE. When the column was disassembled and the Sepharose-bound elastase incubated with the irreversible elastase inhibitor phenyl methane sulfonyl fluoride, additional immunologically identifiable inactive AAT was found in a filtrate suggesting that AAT is a tightly

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bound competitive inhibitor of elastase. Soluble elastase was incubated with AAT in 0.1M Tris, pH 7.6 at a molar ratio of approximately 1:2 (Elastase:AAT). At 1 min. and 60 min., aliquots were withdrawn from the incubation mixture and mixed with an SDS solution. The SDS-PAGE patterns of the 1 min. and 60 min. reaction mixtures showed protein bands with molecular weights of 73,500, 56,900, 54,000, 50,500 and 26,000 daltons. The 54,000 and 50,500 dalton proteins migrate identically with the active AAT applied to the elastase column and the inactive AAT eluted from the elastase column, respectively. The reactants in these gels, therefore, behave similarly to those on the elastase affinity column. These data imply that even in elastase excess the largest degradation which active AAT undergoes is the loss of one or more peptides with a molecular weight of about 3,500 daltons.

Significance:

The observed phenomena support the hypothesis that upon reaction of elastase with active AAT, an enzyme-inhibitor complex was formed at a 1:1 molar ratio. The complex dissociates into the enzyme and a modified form of AAT which has lost a peptide of less than 10% of its original weight. The modified AAT may have been associated with elastase through the carboxy-terminal amino acid of the modified AAT. (See item 10 of renewal application for discussion of this concept.) If this contention can be proven, we can identify the key amino acid at the inhibitory site of elastase on AAT. Since all enzymes inhibited by this site will cleave proteins at this amino acid one might begin the design of drugs to replace AAT in deficient patients with this amino acid and add appropriate inhibiting groups. There are already at least two such methods for tailoring inhibitors of serine proteases to the specificity of the enzyme (5,6).

Studies of the Trypsin Inhibitory Site:

The evolution of the products which develop during the interaction of trypsin and AAT was studied with cellulose acetate membrane (CAM), polyacrylamide gel, and SDS polyacrylamide gel (SDS-PAGE) electrophoretic techniques. We have confirmed Johnson's (7) and Moroi's (8) observations that when trypsin combines with an excess of AAT a single complex (Complex 1) forms and when it combines with an excess of trypsin, two complexes form (Complexes 1 and 2). The second complex has a molecular weight and electrophoretic mobility between the first complex and AAT (Fig. 3). We isolated the first complex on sephadex columns and demonstrated its identity because it contained the radio-labeled trypsin, it reacted against antisera to AAT, and it migrated like the first complex in an electrophoretic field (Fig. 4). Amino acid analysis (Table 1) and molecular weight determination supported Moroi's suggestion that trypsin and AAT combine in a 1:1 ratio but refuted Johnson's suggestion of 2:1 molar binding.

If the AAT-trypsin complex were connected by an ester bond to the serine hydroxyl in trypsin, the bond might be susceptible to degradation in alkaline pH. Treatment of the AAT-trypsin complex with KOH, pH 9.5 for 24 hours at 37°C results in the liberation of active trypsin and AAT with a slightly reduced molecular weight (Fig. 5) and more acidic charge (Fig. 6), suggesting that the AAT-trypsin bond is indeed broken in alkaline solutions. In addition the electrohporetic studies suggest

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that a basic peptide of about 4,000 molecular weight may be lost during the interaction of trypsin and AAT. The amino acid analysis of the isolated complex is deficient in arginine, suggesting that the lost peptide may be basic because of a disproportionate number of arginine residues. These data are all consistent with the hypothesis that the trypsin-AAT complex is an acyl intermediate analogous to the acyl intermediate which serine proteases form with their substrates; however, with AAT the intermediate is stable and does not break down into products (Fig. 10-1 and 10-2, Item 10 of renewal application).

Purification of AAT of ZZ Phenotype by Antibody Affinity Chromatography:

One of the methods proposed to purify AAT of ZZ phenotype was antibody affinity chromatography (p. 11 of grant application). It was suggested that the AAT would have to be eluted with kaotropic ions like thiocyanate (9) and that hydrogen ion elution may not be used because of the pH lability of AAT. However, we have recently determined that AAT has a zone of stability at pH about 2.0 - 2.7 in HCl - KCl, or in glycine - HCl buffers (Table 2). These data now raise the possibility that AAT of ZZ phenotype could be purified with an antibody affinity column and eluted with one of these buffers.

References

1. Gertler, A. and Hofmann, T.: Can. J. Biochem. 48:384 (1970).

2. Erlanger, B.F., Kokowsky, N., and Cohen, W.: Arch. Biochem. 95:271 (1961).

3. Laemmle, U.V.: Nature 227:680 (1970).

4. Hokin, L.E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F., and Perdue, J.F.: J. Bio. Chem. 248:2593 (1973).

5. Thompson, R.C.: Biochem. 12:47 (1973).

6. Thompson, R.C. and Blout. E.: Biochem. 12:51 (1973).

7. Johnson, D.A., Pannell, R.N., and Travis, J.: Biochem. Biophys. Res. Commun. 57:584 (1974).

8. Moroi, M., and Yamasaki, M.: Biochem. Biophys. Acta. 359:130, 1974.

9. Dandliker, W.B., and DeSaussure, V.: Immunochem. LL:139, 1967.

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TABLE 1

Alpha-1-antitrypsin Residues 52000 g		% Moles of Each Amino Acid/Mole Complex[a]			
		Measured	Calculated for 1:1 Molar Complex	Calculated for 1:2 Molar Complex[b]	Complex Predicted 1:X[c]
Lys[d]	34.01	7.60	6.92	6.35	1
His	12.81	2.74	2.49	2.22	1
Arg	9.31	0.00	2.00	1.88	2
Asp[e]	47.89	12.02	11.66	11.73	2
Thr[d]	29.85	6.72	6.24	5.81	1
Ser[d]	24.25	7.73	7.73	8.61	1
Glu	59.12	11.95	12.20	11.27	1
Pro	18.46	4.41	4.31	4.25	1
Gly[d]	26.27	8.32	8.24	9.12	1
Ala[d]	28.80	6.97	7.08	7.18	1
1/2 Cys	2.81	2.28	2.99	3.90	1
Val[d]	26.93	7.20	6.46	6.46	--
Met	7.96	1.40	1.48	1.29	1
Ile	15.62	5.10	4.45	3.66	1
Leu[e]	50.10	10.83	10.50	9.79	1
Tyr	5.84	1.85	2.19	2.56	1
Phe	25.22	5.17	4.58	3.90	1
Trp	not determined	--	--	--	--

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Footnotes to Table 1

a % moles of each amino acid/mole complex was calculated from an amino acid analysis of aliquots of alpha-1-antitrypsin and trypsin which were used to form the complex

b 1:2 complex - 1 mole alpha-1-antitrypsin:2moles of trypsin

c complex predicted on the basis of whether the measured ratio of the amino acid was closer to the ratio predicted for a 1:1 or 1:2 complex. X = moles of trypsin.

d,e residues with greater than 15 and 20 residues difference between 1:1 and 1:2 molar complexes respectively. Therefore these residues would be more likely accurate predictors of the true ratio.

Legend to Table 1

These data show that ratios of 15 of the 17 measured amino acids in the alpha-1-antitrypsin-trypsin complex predict a 1:1 molar ratio. Three residues predicted a 1:2 molar ratio. These data suggest a 1:1 molar complex between alpha-1-antitrypsin and trypsin. The complex has been shown, however, to have lost a peptide of alpha-1-antitrypsin. The two residues which showed a 2:1 molar ratio may have had a disproportionately high ratio in the missing peptide. This formulation would predict that the peptide should be more basic than AAT and that the AAT peptide remaining in the complex should be proportionately more acidic. The increased acidic nature in AAT has in fact been shown by breaking the complex with KOH (Fig. 6). This suggestion is further supported because asp and gly should be the most accurate determinants of molar ratio because they each differ in the predicted 1:1 and 1:2 molar complexes by more than any other residue (27 residues each). This data therefore supports the data from SDS gel electrophoresis which suggests that the peptide has already been lost from the AAT-trypsin complex seen in various electrophoretic techniques and that the peptide which is lost has a peptide with a low proportion of aspartic acid relative to native alpha-1-antitrypsin. and a high proportion of arginine.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

TABLE 2

pH.	2.0	3.0	4.0	5.0	6.0	7.4	9.0	10.0	11.0	12.0
2 hr.	26.4	39.0	39.2	26.0	23.8	25.5	24.2	23.8	23.4	24.9
4 hr.	24.8	35.7	33.8	23.4	22.5	24.8	24.8	25.5	25.7	26.6
6 hr.	25.7	39.8	39.2	25.7	25.7	26.6	26.0	24.1	25.8	26.2
8 hr.	25.4	38.4	39.6	27.3	23.8	22.8	23.9	24.1	23.6	25.3
24 hr.	27.8	42.4	41.9	27.3	26.0	28.9	27.0	27.0	27.9	26.6

Standard Curve Day I

2 µg 6.6
5 µg 19.6
10 µg 40.6

Standard Curve Day II

2 µg 8.7
5 µg 19.0
10 µg 38.5

pH Stability of Alpha-1-antitrypsin.

Lyophilized, purified AAT was placed in low ionic strength buffer at the specified pH and incubated at 25°C for the specified time. The pH was then raised to 7.6, 10 µg of trypsin were added and the trypsin activity was measured. Results are expressed as moles of substrate hydrolyzed/min. X 10^7. Higher enzyme rates indicate low inhibitory activity.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

10. Plan for the Coming Year:

From the work in our laboratory during the past year, a hypothesis can be proposed for the biochemical mechanism of AAT and the hypothesis can be subjected to experimental tests. Earlier, we proposed that AAT interacted with enzymes in a manner similar to their interaction with protein substrates but instead of cleaving the substrate, AAT and the enzyme formed a stable intermediate. The interaction of serine proteases and substrates has been schematized by Stroud (Fig. 10-1). The schematic shows the different intermediates which develop during the interaction of serine proteases with substrates. Recently, Moroi (Biochem. Biophys. Acta. 359:130, 1974) has suggested that the AAT-enzyme complex is one of these intermediates, called the acyl intermediate (Panel 4, Fig. 10-1). In this intermediate the peptide on the amino-group side of the peptide cleavate has fallen from the complex and the serine hydroxyl at the enzyme catalytic site is bound to the carboxyl group of the amino acid at the end of the remaining protein fragment. This bond is an ester bond, and as mentioned in the progress report these bonds are often susceptible to cleavage under mild alkaline conditions. Our work in the past year has shown that the AAT in the AAT-enzyme complex has already lost a peptide and that the loss of the peptide is not dependent on unbound or active trypsin in the solution. In addition, the bond between the AAT and trypsin is broken under mild alkaline conditions. These data are highly suggestive that the AAT-enzyme complex is indeed an acyl intermediate (Fig. 10-2).

During the ensuing year we wish to test the next step in the hypothesis: Enzymes are attached by an alkaline labile bond to the new carboxy terminal amino acid on AAT. The design of the experiments are diagramed in Figure 10-3.

We have already determined that KOH can cleave the AAT-trypsin complex into an active enzyme and a fragment of AAT with a small peptide missing. If the enzyme is bound to the carboxy-terminal amino acid through its carboxyl group, as it must be to fulfill the requirements of the hypothesis, then the AAT in the complex will have no chemically recognizable carboxy-terminal amino acid. Carboxypeptidases are enzymes which cleave amino acids from the carboxy-terminal end of proteins, one residue at a time. Therefore, if the AAT-enzyme complex is an acyl intermediate and if a carboxypeptidase of appropriate specificity is added to the complex, the carboxy-terminal amino acid of the enzyme-bound fragment of AAT will not be removed (Fig. 10-3, left arrows). However, if the complex if first dissociated and then the carboxypeptidase is added, the amino acid at the enzyme inhibitory site will be removed and determined in the amino acid analyzer (Fig. 10-3 right arrows).

The experiment can be further illustrated by describing its use in determining the amino acid at the inhibitory site on AAT for trypsin. Carboxypeptidase B (CPB) is an enzyme which cleaves arginine and lysine amino acids from the carboxy-terminal end of proteins and trypsin cleaves proteins at arginine and lysine residues inside of the peptide chain. Therefore, a solution containing AAT-trypsin complexes will be divided into two aliquots and one aliquot will be dissociated with KOH. Then both aliquots will be treated with CPB. Amino acids which are released will be analyzed on an amino acid analyzer. If trypsin is bound to carboxy-terminal arginine or lysine on the remaining AAT peptide, then an arginine or lysine should be removed only from the complex dissociated in KOH. This experiment would have the dual function of demonstrating that trypsin is bound to the carboxy-terminal amino acid of the remaining peptide, and identifying this key amino acid. All enzymes inhibited by this

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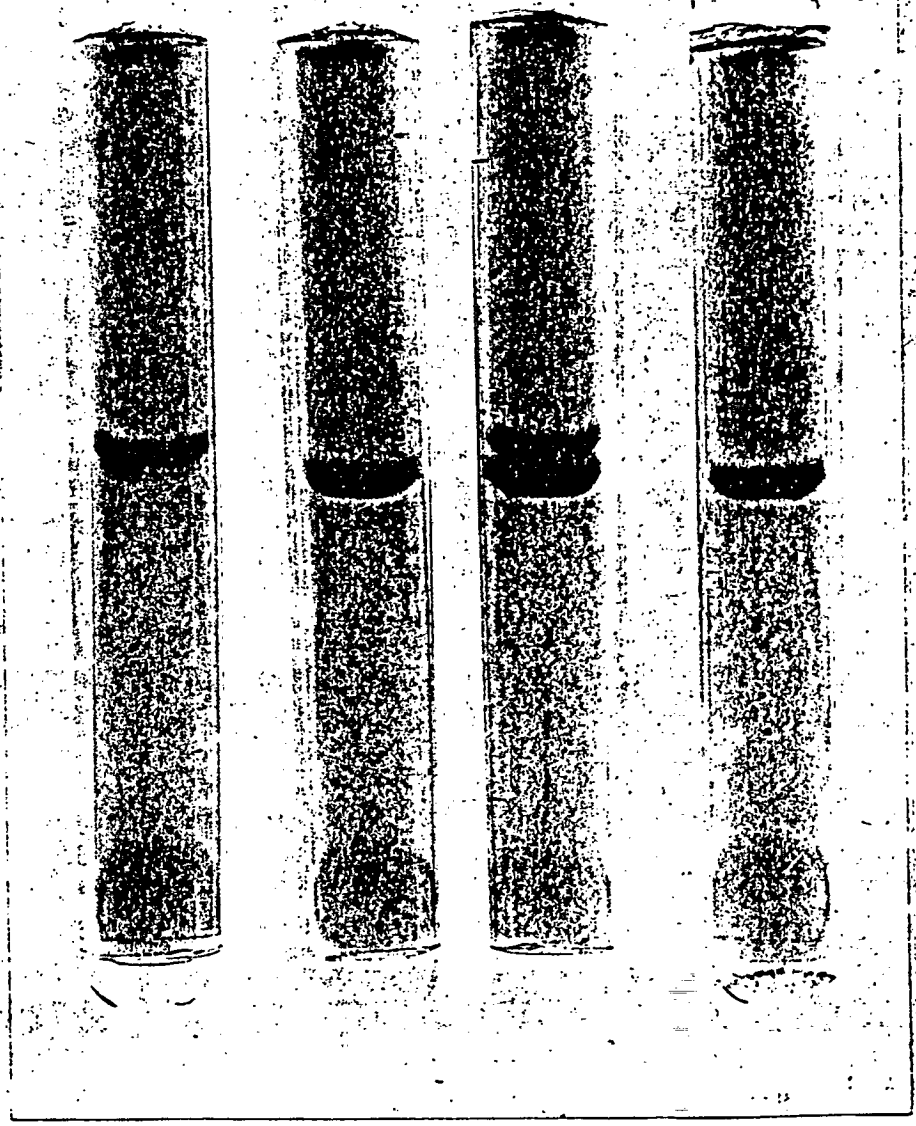

Figure 2. The SDS-gel electrophoretic patterns of active AAT (reduced; gel 1), inactive AAT (reduced; gel 2), mixture of active and inactive AAT (reduced; gel 3), and inactive AAT (non-reduced; gel 4). The separating gel was 9% total acrylamide. The stacking gel was 3.08% total acrylamide. The electrode buffer was tris-gly, pH8.3 containing 0.1% SDS. Electrophoresis was conducted at room temperature for 4 hours. A constant current of 3.0 ma per gel was applied. Migration is from top (Cathode) to bottom (Anode).

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Figure 3 A:
Cellulose Acetate Membrane Electrophorogram of AAT and Trypsin: A. AAT is in molar excess of trypsin. B. Trypsin is in molar excess of AAT. C. Native AAT control. A black string is stretched below the colplex formed in the presence of AAT excess and above the complex formed in trypsin excess. Trypsin has migrated off the strip in an anodal direction. (The anode is at the bottom and the cathode is at the top.)

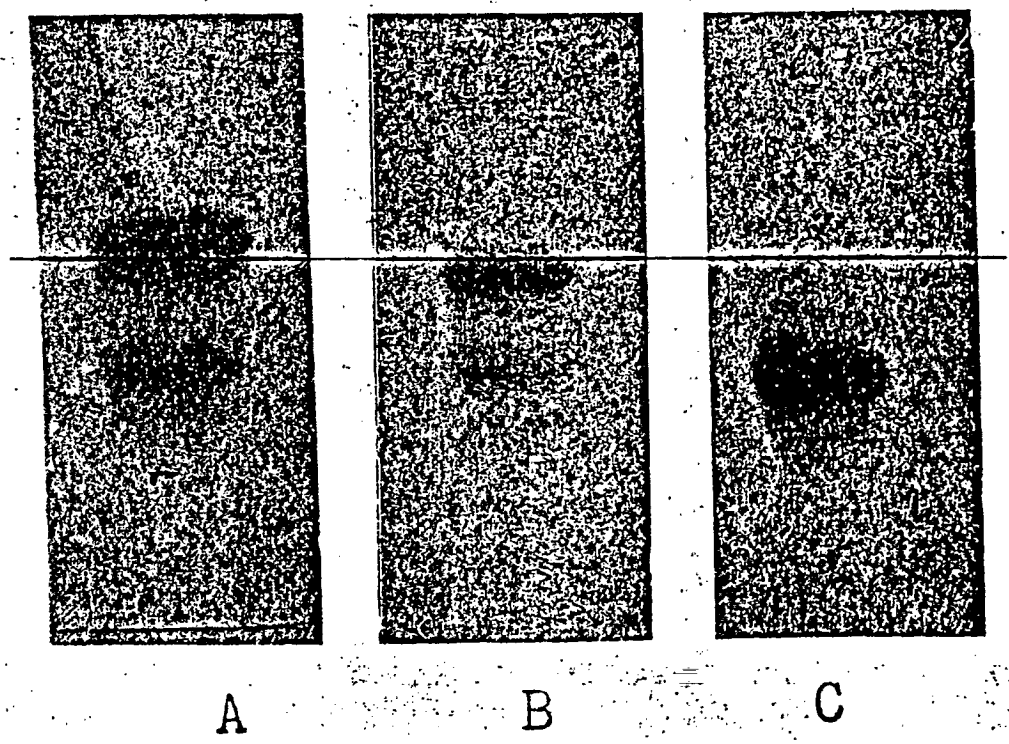

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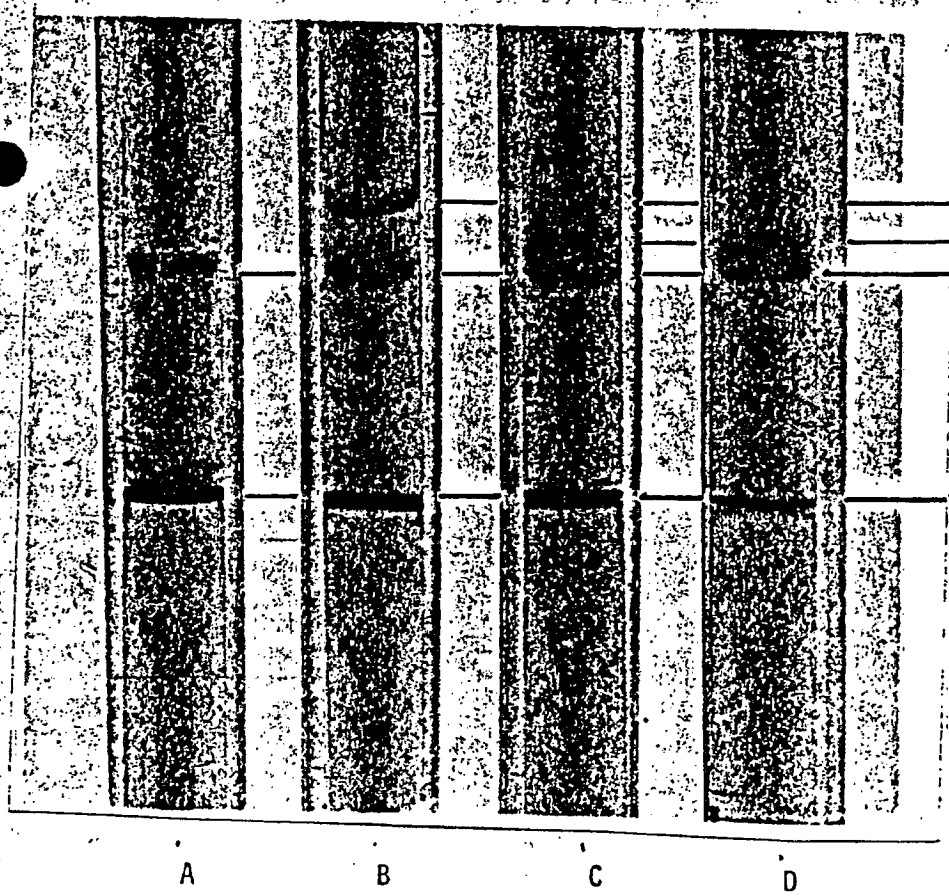
Figure 3 B:
Sodium Dodecyl Sulfate-Poyacrylamide Gel Electrophorogram of the Interaction of AAT and Trypsin with a Molar Excess of Trypsin.

A. AAT and Trypsin were added to sodium dodecyl sulfate (SDS) before mixing them together. No complex forms and therefore, this gel serves to locate native AAT and Trypsin as controls.

B. Run after reactants incubated at room temperature for one minute. A 73,000 dalton complex is present.

C. Run after reactants incubated at room temperature for 30 minutes. In addition to the 73,000 dalton complex, an additional complex of 62,000 daltons has formed. This complex does not form when AAT is in molar excess and in the presence of trypsin inhititors added at one minute. It is therefore, dependant on free active trypsin to form it. Data indicate that it represents the 73,000 dalton complex minus a 12,000 dalton peptide ocleaved form the bound trypsin.

D. Run after reactants incubated at room temperature for 60 minutes. The 73,000 dalton complex has been completely converted into the 62,000 dalton complex.



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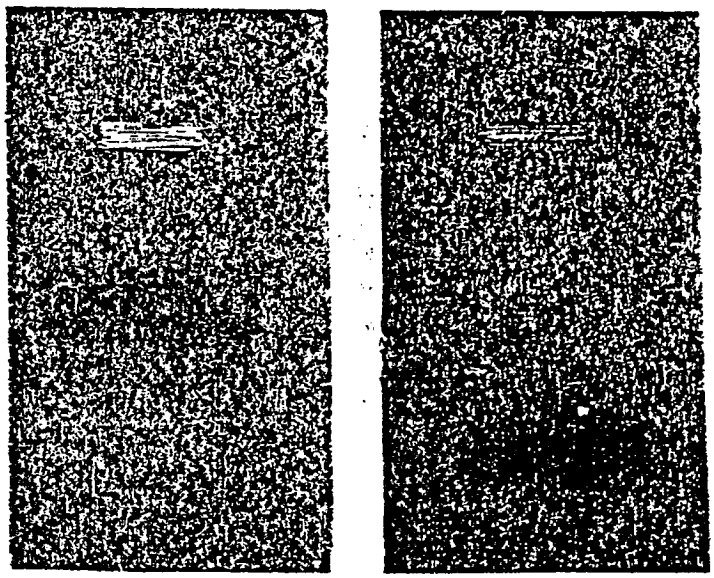
Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Figure 4:
Cellulose Acetate Membrane Electrophoregram of AAT-trypsin Complex Formed in AAT excess and Isolated on a Sephadex Column: A. Isolated complex. B. Native AAT control. The isolated complex contained radioactivity from the I-125 labeled trypsin and reacted in double-immunodiffusion of Ouchterlony to give a precipitin line between the isolated complex and native AAT.



A B

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Figure 5:

Polyacrylamide Gel Electrophorogram in Sodium Dodecyl Sulfate to Study the Effects of KOH and Phenyl Methane Sulfonyl Fluoride (PMSF) on the Interaction between AAT and Trypsin.

In samples A-D there was a 2:1 molar excess of AAT:trypsin. The AAT and trypsin were combined and incubated for 1 minute. Then PMSF was added to samples A and B. After 15 minutes, the pH was raised to 9.5 with KOH in samples C and D. Sample E contained only fresh native AAT and Sample E contained only native trypsin. These SDS-PAGE showed that PMSF had no influence on complex formation (A). The 73,000 molecular weight complex forms when excess AAT is added to trypsin (D). When these reactants are incubated in KOH at 37°C for three hours, an additional complex (62,000 daltons) is seen (C). This complex has been shown to develop as a result of the cleavage of a large peptide from the bound trypsin by free trypsin; therefore, the KOH has released active trypsin into the reaction mixture. In addition, the AAT band is split, indicating that the released AAT has lost a small peptide of about 4000 daltons. Free trypsin is not seen in gel C because active trypsin incubated for several hours is broken down into peptides which are not recognizable on these gels. When KOH is added after PMSF the released trypsin is inactivated by the PMSF. Therefore, the 62,000 dalton complex is not seen and a trypsin band is seen. In addition, the low molecular weight AAT band is still present, showing that the appearance of this AAT cleavage is not dependant on the free active trypsin, but is the AAT which was released from the complex. These data suggest, therefore, that KOH has dissociated the complex into active trypsin and AAT which has lost a peptide of about 4000 daltons.

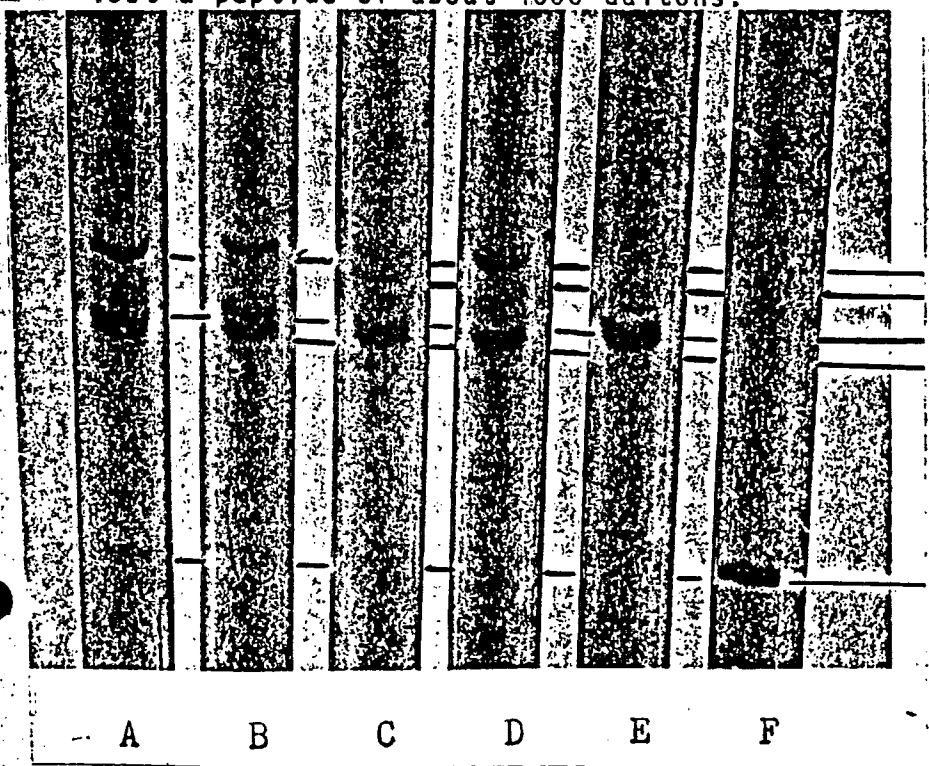

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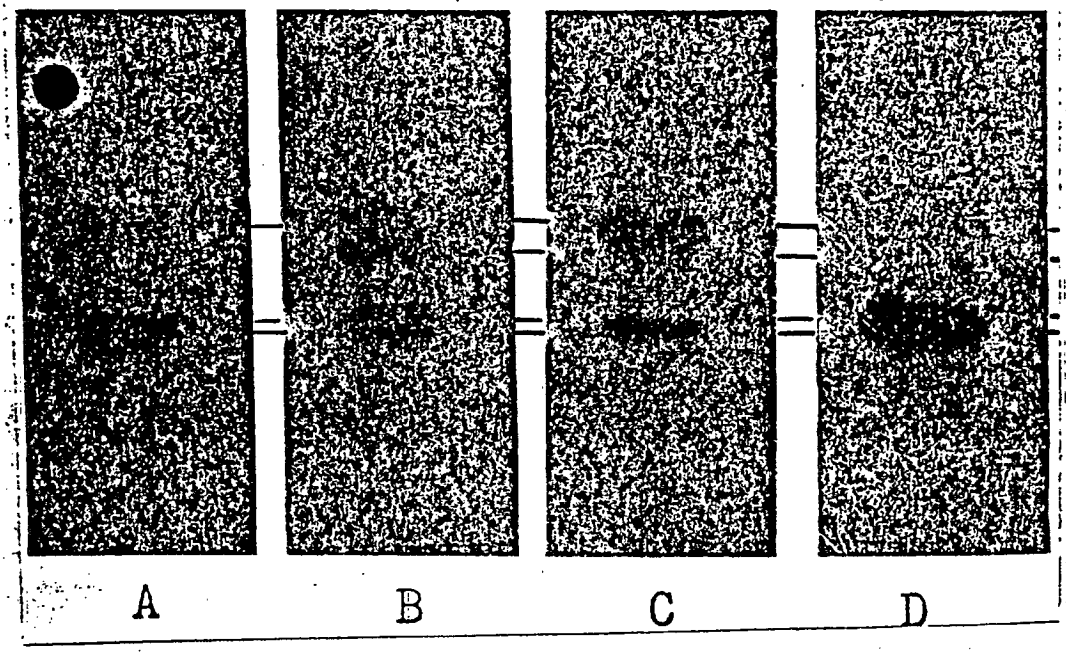
Figure 6:

Cellulose Acetate Electrophorogram to study the Effects of KOH and Phenyl Methane Sulfonyl Fluoride (PMSF) on the Interaction Between AAT and Trypsin.

In samples A-C there was a 2:1 molar excess of AAT:trypsin. The experiment was performed as described in Figure 3. Sample A contained PMSF and KOH, B contained KOH only, C did not have either KOH or PMSF. In electrophorogram "C" one band is formed by the interaction of AAT and trypsin. In electrophorogram "B" the complex band,seen at the top is split. This pattern is seen when excess free trypsin is present and therefore, indicates that free trypsin has been liberated by dissociating the AAT-trypsin complex. The splitting of the lower band indicates that AAT released from the complex is more acidic than native AAT (D) and has therefore, lost a peptide which is basic relative to native AAT. In electrophorogram "A",PMSF, a trypsin inhibitory, has prevented the appearance of the split band at the top,again, indicating that this band requires the presence of active trypsin. However, the AAT with more anodal migration is evident because of the persistence of active trypsin. However, the AAT with more anodal migration is is unaffected by the presence of PMSF as seen by the persistence of the split band at the bottom.

(The anode is at the bottom).



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#1022 EGLE

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

January 30, 1975

Grant Application No. 1022

PULMONARY

To: The committee conprising Drs. Gardner, Jacobson and Liebow

Subject: John L. Egle, Jr., Ph.D., Medical College of Virginia, Richmond
New application No. 1022
"Respiratory retention and effects on pulmonary funcation of constituents of the vapor phase of cigarette smoke"

History

This new application was not previously screened as a case.

Request

Application No. 1022 requests $29,813 for the first year of a two year study.

Documents submitted (attached)

1. Application dated January 24, 1975 (17 pages, including C.V. of Dr. Egle).

2. Six reprints

Comment

It will be noted from the enclosed reprints that Egle has had support from the American Medical Association Education and Research Foundation.

D.S.

DS:wg
Encl.

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#1022

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JAN 2 9 1975

Application for Research Grant Date: 1-24-75

(Use extra pages as needed).

1. Principal Investigator (give title and degrees):

John L. Egle, Jr., Ph.D.
Associate Professor of Pharmacology

2. Institution & address:

Medical College of Virginia
Department of Pharmacology
Box 726
Richmond, VA 23298

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Respiratory retention and effects on pulmonary function of constituents of the vapor phase of cigarette smoke.

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: Two years

7. Brief description of specific research aims:

1. Complete a study of the respiratory tract retention of constituents of the vapor phase of cigarette smoke.

   A. Study the effect of ventilatory rate, tidal volume and concentration inhaled on retention of these constituents.

   B. Study the retention of these compounds by the total respiratory tract and by the upper and lower portion.

2. Study the effects of all major vapor phase constituents on pulmonary function. Parameters to be measured will include:

   A. Ventilatory rate
   B. Tidal volume
   C. Pulmonary resistance
   D. Pulmonary compliance
   E. Transpulmonary pressure and intrapleural pressure as a means of determination of pulmonary resistance and pulmonary compliance.

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Compounds to be studied will include:

Part 1: Nitric oxide, hydrogen cyanide, methyl chloride, acetonitrile and furan.

Part 2: Acetaldehyde, formaldehyde, acrolein, isoprene, acetone, ammonia, methanol, benzene, toluene and the compounds indicated for study in Part 1.

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8. Brief statement of working hypothesis:

More than 50 compounds are known to occur in the vapor phase of cigarette smoke at a concentration of one microgram or more per 40 ml puff.[1] It is proposed that the respiratory tract retention be measured in experimental animals of compounds not previously studied in this manner and known to occur in relatively high concentrations or considered to be noxious substances. Examples of such substances include nitric oxide, hydrogen cyanide, methyl chloride, acetonitrile and compounds in the furan series. A second objective would be to systematically study the effects of these compounds, as well as those previously studied in terms of retention, on such parameters of respiratory function as rate, tidal volume, pulmonary resistance, pulmonary compliance, and intrapleural pressure. Such studies would permit comparison of the capacity of individual smoke constituents to alter respiratory function. Since the substances proposed for study are brought into contact with the respiratory tract during smoking, it would seem desirable to know as much as possible about their handling by the respiratory tract in terms of both total respiratory tract retention as well as fractional retention by subdivisions of the respiratory tract. It would also be useful to determine the concentrations of the substances actually reaching the lower tract when a given concentration is inhaled. We have obtained this type of information for several compounds.[2-7] More recently toluene and benzene have been studied.

~~9. Details of experimental design and procedures (append extra pages as necessary)~~

It would also appear very desirable to know more about the ability of these substances to alter pulmonary function. Aviado[8] and other investigators[9-10] have studied the effect of cigarette smoke on pulmonary resistance and compliance and have reported changes in these parameters. The experimental conditions and results of these studies have varied considerably. Considerable attention has been devoted to the effects of such major air pollutants as NO_2 and SO_2 on pulmonary function.[11-12] However, it appears that there has been no systematic effort to determine and compare the effects, if any, on pulmonary function of important individual constituents of cigarette smoke at concentrations which would be encountered during smoking. This type of study would also permit identification of the components responsible for any changes of this type attributed to whole cigarette smoke. The proposed study is designed to answer these questions.

9. Details of experimental design and procedures:

1. Respiratory retention of vapor phase constituents

Vapors of each pure substance dispersed in air will be studied individually. Concentrations approximating those reported for each compound in the gas phase of cigarette smoke will be employed. Appropriate amounts of each substance to give the desired concentration when diluted will be introduced into a 13.5 liter recording respirometer. The respirometer will contain mineral oil as an insulating substance and a fan will be inserted in the system for circulating the vapor. The compounds studied thus far have been quite stable in such a system, therefore it seems reasonable to assume it would be satisfactory for the other vapors. The animal will be anesthetized and connected to the system by means of a mask or endotracheal tube and permitted to inhale vapor from the respirometer. The direction of vapor flow will be controlled by two-way valves. The animal will inhale vapor from the respirometer and exhale into a collecting bag. Such a system keeps the concentration inhaled constant. By taking samples of the vapor in the respirometer and collecting bag to determine the concentration of each and by recording the volumes inhaled and exhaled, it is possible to calculate the amount retained and the percentage of that inhaled which is retained.

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Using this system it is possible to measure retention of the vapors being studied under varying conditions of ventilatory rate, tidal volume, and concentration inhaled. Efforts will constantly be made to ensure that there is no loss of vapor from the system which might erroneously be interpreted as respiratory tract uptake. This would include conducting sham experiments and making any necessary corrections in results obtained in inhalation experiments. This would include taking into account all dead space in the system.

In addition to retention of each vapor by the entire respiratory tract, another major aim will be to determine the amount taken up by different segments of the respiratory tract. This can be accomplished by exposing isolated portions of the respiratory tract. Upper tract retention will be measured by severing the trachea at its lower end and passing vapor through the nose using a face mask and collecting it from the trachea. Upper tract experiments will be conducted on a "one-way" or "two-way" basis. In the one-way experiments the vapor will be passed into a collecting bag at the lower end of the trachea. A two-liter syringe will be attached to the end of the trachea for the two-way experiments, with the vapor being drawn into the syringe, then returned upward and expelled through the nose, finally being collected in a bag after passing through a two-way valve.

The two-way upper tract experiments will be carried out to determine the amount of compound under investigation taken up by this region during a complete ventilatory cycle. The one-way upper-tract experiments do not simulate any type of normal ventilation. The purpose of these experiments will be to measure the reduction in concentration of the inhaled vapor between the nose and bronchioles. Information of this type would be of value in the design and interpretation of experiments involving nasal inhalation and also in studies dealing with effects on lower tract tissue using isolated organ or in vitro systems. The one-way experiments will indicate the amounts of any substance removed by the upper tract and also provide a determination of the concentration actually reaching the lower tract. The two-way experiments would permit measurement of the amount taken up by this portion of the respiratory tract during a complete ventilatory cycle.

Lower tract retention will be measured by inserting an endotracheal tube thereby restricting the area of exposure to the lower portion of the respiratory tract. Otherwise, the techniques are similar to the total tract experiments.

All of these experiments will be of short duration, 1-3 minutes, and will involve the inhalation of a total of 1-3 liters of the vapor being studied.

2. Effects of vapor phase constituents on pulmonary function

Procedures for measurement of pulmonary function

Ventilatory rate, tidal volume, and minute volume are simple but important aspects of pulmonary function that can be easily measured with a respirometer.

To measure pulmonary resistance and pulmonary compliance, we will utilize a modified exposure chamber to simulate a plethysmograph. A schematic diagram of the apparatus is shown in figure 1. The dogs will be anesthetized with

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30 mg/kg pentobarbital sodium. An endotracheal tube will be installed to limit airway exposure to the mouth opening only. Placed onto the endotracheal tube will be an adaptor fixed by a connector. The distal side of the adaptor will be attached to a pneumotachograph connected to a differential transducer (1) to measure air flow ($\dot{v}$)(pressure drop across the pneumotachograph). Into the right interpleural space a rubber catheter will be permanently secured. The opening of the catheter will be connected to a Statham differential transducer (2). This transducer will also be connected to a lead from the adapter. The catheter will be used to measure the intrapleural pressure, the adapter used to measure the open or mouth airway pressure. These two pressure readings will be, with the use of the transducer, represented as one reading known as the transpulmonary pressure. A third transducer (3) will be used to measure the pressure differential between the chamber and the ambient air. This measurement is a component of pulmonary resistance. The signals from the three transducers will be fed into a Tektronix S103N oscilloscope. For our purposes, we will have three channels in operation, two horizontal and one vertical. A picture of each set of experiments will be taken and developed. The pictures will be used to plot the tangent of the graphs. From the loops on the oscilloscope the pulmonary resistance and compliance can be calculated using the tangent of the loop over its linear range by the method described by Mead and Wittenburg.[13] Measurement of end tidal CO_2 levels will also be made during these experiments.

Acute inhalation

The animals will be prepared for measurement of pulmonary function as described. Vapors of each compound to be studied will be generated by passing air through the substance. This concentration vapor will be transferred to a recording respirometer. The respirometer will serve as a reservoir for the vapor and also permit monitoring of the respiratory pattern during the exposure period. The animals will inhale the vapor for a varying period ranging from one inhalation to at least three minutes. (Aviado[8] has reported that one puff of cigarette smoke is sufficient to produce an immediate increase in pulmonary resistance.) Depending upon the initial experience (response or lack of response) with each compound, the exposure period will be adjusted to obtain a clear picture of the relationship between exposure time and alteration of pulmonary function for each substance.

Chronic inhalation

For these experiments, dogs will be placed in large (approximately 300 liter) chambers and exposed to each of the vapors being investigated. Exposure will be based on the assumption that a heavy smoker would be exposed to approximately 400 inhalations of smoke (and therefore the chemicals being studied) each day. Thus, the dogs will be allowed to inhale the vapor about 400 times or for a period of 20 to 30 minutes (depending on the ventilatory rate). Dogs will be exposed 5 days a week. Shorter or longer periods of exposure may also be selected depending upon the results with each compound. A dynamic system will be used for exposure. A vapor of the compound to be studied will be generated by continuous air flow through the substance. This vapor will then be mixed with air to achieve the proper concentration as it flows into a reservoir. The vapor will be drawn from the reservoir through the exposure chamber at a constant flow

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rate. A negative pressure system will be employed with a vacuum pump pulling the vapor into the chambers with flow controlled by a critical orifice. The concentration of the compound being studied in the chamber will be monitored by periodic sampling and analysis by gas chromatography.

Periodically, each animal will be placed in the plethysmograph and measurements of pulmonary function performed. Such tests will be carried out on each animal at 7 day intervals during the exposure period. Animals will continue to be tested after the period of exposure, particularly if alterations in pulmonary function are observed and if they persist beyond the period of exposure. Thus, the duration of any changes produced under these conditions will be determined or it will be established that they persist until a certain point after which measurements are no longer made (e.g., six months). It would appear that the exact design of these chronic experiments, particularly in regard to duration, would depend upon the magnitude and duration of effects observed with each compound.

Animals

It is anticipated that all experiments will be performed with mongrel dogs. Such animals have been used in the studies carried out involving respiratory retention of vapor phase constituents. Little variability has been observed between dogs for a given set of experimental conditions and it has been possible to pool the data for all animals within a particular experimental series. It is conceded that there would be some advantages in using purebred beagles, but these animals would be approximately ten times more expensive and it is doubted that the benefits of using such animals outweigh the considerable cost involved.

Chemical Analysis

The compounds studied thus far have been measured by either spectrophotometry (all aldehydes, acetone and ammonia) or gas chromatography (isoprene, methanol, benzene, toluene and furan). The fact that we have experience with and established methodology for measuring very low concentrations of these compounds would facilitate the initiation of experiments involving tests of pulmonary function. Possibly all of these compounds would be analyzed by gas chromatography in the future. The compounds not previously studied would also be measured by gas chromatography although techniques (selection of column, etc.) would have to be devised for them.

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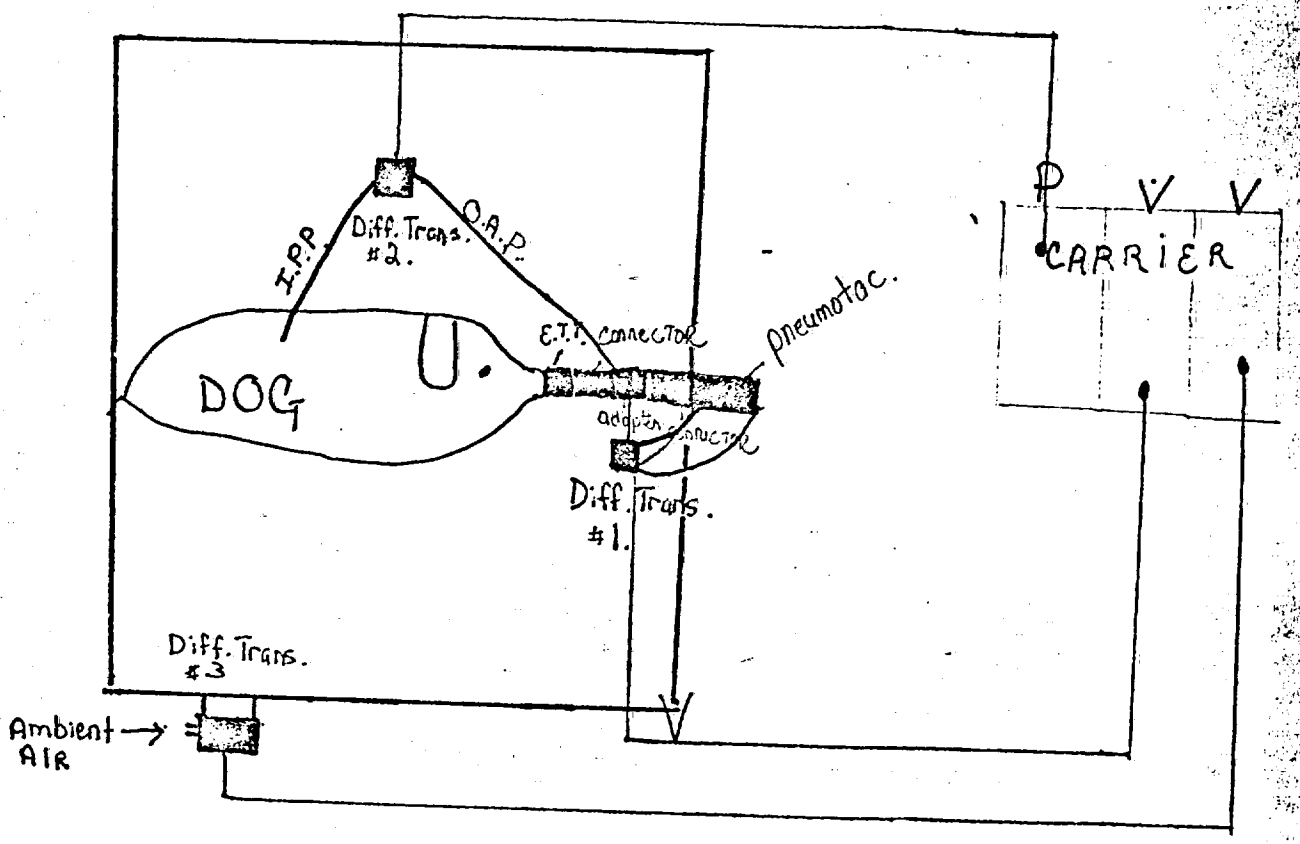
Fig 1



I.P.P. - Intra pleural pressure
E.T.T. - Endotracheal tube
O.A.P.. - Open Airway Pressure
Pneumotac - Pneumotachograph
Diff. Trans. - Differential transducer

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References

1. Newsome, J. R., Norman, V. and Keith, C. H.: Vapor phase analysis of tobacco smoke. Tobacco Sci. 9:102-110, 1965.

2. Egle, J. L., Jr.: Retention of inhaled acetaldehyde in man. J. Pharmacol. Exp. Ther. 174:14-19, 1970.

3. Egle, J. L., Jr.: Retention of inhaled acetaldehyde in the dog. Arch. Environ. Health 24:354-57, 1972.

4. Egle, J. L., Jr.: Retention of inhaled formaldehyde, propionaldehyde and acrolein in the dog. Arch. Environ. Health 25:119-124, 1972.

5. Egle, J. L., Jr.: Retention of inhaled acetone and ammonia in the dog. Amer. Ind. Hyg. Asso. J. 34:533-39, 1973.

6. Egle, J. L., Jr.: Respiratory retention of isoprene and methanol in the dog. Toxicol. Appl. Pharmacol. 25:470-471, 1973.

7. Egle, J. L., Jr. and Gochberg, B.: Respiratory retention of toluene and benzene in the dog. The Pharmacologist 16:299, 1974.

8. Aviado, D. M. and Palecek: Pulmonary effects of tobacco and related substances I. Pulmonary compliance and resistance in the anesthetized dog. Arch. Environ. Health 15:187-193, 1967.

9. Eich, R. H., Gilbert, R. and Auchincloss, J. H., Jr.: The acute effect of smoking on the mechanics of respiration in chronic obstructive pulmonary emphysema. Amer. Rev. Tuberc. 76:22-23, 1957.

10. Atlinger, E. O., Goldstein, M. M. and Segul, M. S.: Effects of smoking upon the mechanics of breathing. Amer. Rev. Tuberc. 77:10-16

11. Lewis, T. R., Campbell, K. I. and Vaughan, T. R., Jr.: Effects on canine pulmonary function. Arch. Environ. Health 18:596, 1969.

12. Vaughan, T. R., Jr., Jennelle, L. F. and Lewis, T. R.: Long-term exposure to low levels of air pollutants: Effects on pulmonary function in the beagle. Arch. Environ. Health 19:45-50, 1969.

13. Mead, J. and Whittenberger, J. L.: Physical properties on human lung measured during spontaneous respiration. J. Appl. Physiol. 5: 779-796, 1953.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Facilities for conducting this study are available in the Department of Pharmacology. This includes laboratories for performing the experiments and animal quarters. Available equipment includes a Beckman GC 72-5 gas chromatograph, a Beckman DU-2 spectrophotometer, two Collins 13.5 liter respirometers, a Harvard respirator, two exposure chambers, a Tektronix S103N oscilloscope, an Instrumentation Association Capnograph, and a six channel Grass Model 7 polygraph.

11. Additional facilities required:

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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12. Biographical sketch of investigator(s) and other professional personnel:

NAME:	John Lee Egle, Jr.
BIRTH DATE:	July 13, 1939
BIRTH PLACE:	Martinsburg, West Virginia
CITIZENSHIP:	United States
SOCIAL SECURITY NUMBER:	234-70-1680
MARITAL STATUS:	Married; Two Children
HOME ADDRESS:	8114 Duncaster Road, Richmond, Virginia 23235
OFFICE ADDRESS:	Department of Pharmacology, Medical College of Virginia Richmond, Virginia 23298

EDUCATION:

B.S.	Shepherd College	Shepherdstown, West Virginia	1961
M.S.	West Virginia University	Morgantown, West Virginia	1963
Ph.D.	West Virginia University	Morgantown, West Virginia	1964

POSTDOCTORAL TRAINING:

Cardiovascular Training Program - Bowman Gray School of Medicine

Winston-Salem, North Carolina (1964-1966)

ACADEMIC APPOINTMENTS:

Department of Pharmacology Medical College of Virginia Richmond, Virginia 23298	Assistant Professor	1966-1974
Department of Pharmacology Medical College of Virginia Richmond, Virginia 23298	Associate Professor	1974-

MEMBERSHIP-SCIENTIFIC SOCIETIES:

ASPET

Society of Toxicology

Sigma Xi

AAAS

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PUBLICATIONS

Egle, John L., Jr., Meredith, Jessee H. and Little, J. Maxwell:

The effect of dichloroisoproterenol, pronethalol and propranolol on oxygen consumption in the rat.
J. Pharmacol. Exp. Ther. 158: 399-404, 1967.

Meredith, Jessee H., Little, J. Maxwell and Egle, John L., Jr.:

A comparison of simultaneous cardiac output measurements by two methods: the Oxygen-Fick principle and the electromagnetic flow-meter.
Amer. Surgeon 33: 949-52, 1967.

Egle, John L., Jr.:

Retention of inhaled acetyladehyde in man.
J. Pharmacol. Exp. Ther. 174: 14-19, 1970.

Egle, John L., Jr.:

Single breath retention of acetaldehyde in man.
Arch. Environ. Health 23: 427-433, 1971.

Egle, John L., Jr.:

Retention of inhaled acetaldehyde in the dog.
Arch. Environ. Health 24: 354-357, 1972.

Egle, John L., Jr.:

Retention of inhaled formaldehyde, propionaldehyde and acrolein in the dog.
Arch. Environ. Health 25: 119-124, 1972.

Egle, John L., Jr.:

Effects of inhaled acetaldehyde and propionaldehyde on blood pressure and heart rate.
Toxicol. Appl. Pharmacol. 23: 131-135, 1972.

Egle, John L., Jr., Putney, James W., Jr. and Borzelleca, Joseph F.:

Effects of haloalkane propellants on cardiac rate and rhythm in mice.
JAMA 222: 786-789, 1972.

Egle, John L., Jr., Hudgins, Patricia M., and Lai, Fong M.:

Cardiovascular effects of intravenous acetaldehyde and propionaldehyde in the anesthetized rat.
Toxicol. Appl. Pharmacol. 24: 636-643, 1973.

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PUBLICATIONS

Egle, John L.,Jr.:

Retention of inhaled acetone and ammonia in the dog.
Amer. Ind. Hyg. Ass. J. 34:533-39, 1973.

Egle, John L.,Jr. and Hudgins, Patricia M.:

Dose dependent sympathomimetic and cardioinhibitory effects of acrolein and formaldehyde in the anesthetized rat.
Toxicol. Appl. Pharmacol. 28:358-66, 1974.

Beckner, Jacqueline S., Hudgins, Patricia M. and Egle, John L.,Jr.:

Effects of acetaldehyde, propionaldehyde, formaldehyde and acrolein on contractility, ^{14}C-norepinephrine and ^{45}Calcium binding in isolated smooth muscle.
Res. Commun. Chem. Pathol. Pharm. 9:471-88, 1974.

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ABSTRACTS

Egle, John L., Jr.:

Egle, J. L., Jr. and Saxe, L. H.:

Quantitative changes in the cecal flora and fauna of rats treated with antibiotics.
J. Parisitol. 49: 16, 1963.

Egle, J. L., Jr.:

Quantitative studies of antibiotic action upon the cecal flora and fauna of rats.
Fed. Proc. 23: 512, 1964.

Egle, J. L., Jr.:

Retention of inhaled acetaldehyde in man.
The Pharmacologist 10: 174, 1968.

Egle, J. L., Jr.:

Single breath human retention of inhaled acetaldehyde.
The Pharmacologist 12: 265, 1970.

Egle, J. L., Jr.:

Respiratory uptake of acetaldehyde and propionaldehyde in the dog.
The Pharmacologist 13: 273, 1971.

Hudgins, P. M., Egle, J. L., Jr. and Lai, F. M.:

Cardiovascular effects of acetaldehyde.
The Pharmacologist 13: 298, 1971.

Egle, J. L., Jr., Putney, J. W., Jr. and Borzelleca, J. F.:

Cardiac function in mice following exposure to haloalkane propellants alone and in combination with bronchodilators.
Toxicol. Appl. Pharmacol. 22: 306, 1972.

Egle, J. L., Jr.:

Respiratory uptake of formaldehyde, propionaldehyde and acrolein in the dog.
Fed. Proc. 31: 518, 1972.

Egle, J. L., Jr.:

Pressor effects of four aliphatic aldehydes and their interactions with ^{14}C-norepinephrine in an isolated smooth muscle preparation.
Fed. Proc. 32: 795, 1973.

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ABSTRACTS

Egle, J. L., Jr.:

Respiratory retention of acetone and ammonia in the dog.
Toxicol. Appl. Pharmacol. 25: 470-471, 1973.

Egle, J. L., Jr.:

Respiratory retention of isoprene and methanol in the dog.
Fed. Proc. 33: 569, 1974.

Egle, J. L., Jr. and Gochberg, B.:

Respiratory retention of toluene and benzene in the dog.
The Pharmacologist 16: 299, 1974.

Green, M. A. and Egle, J. L., Jr.:

LD_{50} determination and hypotensive effect of 3-methyl-2-benzothiazolone hydrazone.
Toxicol. Appl. Pharmacol. (in press)

Egle, J. L., Jr., Borzelleca, J. F. and Long, J.E.:

An evaluation of the cardiac sensitizing potential of Scotchgard.
Toxicol. Appl. Pharmacol. (in press)

Green, M. A. and Egle, J. L., Jr.:

Effects of intravenous acetaldehyde, acrolein, formaldehyde and propionaldehyde on arterial blood pressure following acute guanethidine treatment.
Fed. Proc. (in press)

Gochberg, B. J. and Egle, J. L., Jr.:

Retention of inhaled furan in the dog.
Fed. Proc. (in press)

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13. Publications: (five most recent and pertinent of investigator).

Egle, John L., Jr.:
Retention of inhaled acetyladehyde in man.
J. Pharmacol. Exp. Ther. 174: 14-19, 1970.

Egle, John L., Jr.:
Retention of inhaled acetaldehyde in the dog.
Arch. Environ. Health 24: 354-357, 1972.

Egle, John L., Jr.:
Retention of inhaled formaldehyde, propionaldehyde and acrolein in the dog.
Arch. Environ. Health 25: 119-124, 1972.

Egle, John L., Jr., Hudgins, Patricia M., and Lai, Fong M.:
Cardiovascular effects of intravenous acetaldehyde and propionaldehyde in the anesthetized rat.
Toxicol. Appl. Pharmacol. 24: 636-643, 1973.

Egle, John L., Jr.:
Retention of inhaled acetone and ammonia in the dog.
Amer. Ind. Hyg. Asso. J. 34: 533-539, 1973.

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
John L. Egle, Jr., Ph.D. Associate Professor of Pharmacology	50	
Technical		
Bethe Gochberg Lab Specialist B	100	$10,512
Mark Green Lab Technician	50	4,000
Benefits		1,460
Sub-Total for A		$15,972

B. Consumable supplies (by major categories)	
Chemicals	500
Glassware	500
Animals	2,500
Animal Care	1,250
Sub-Total for B	4,750

C. Other expenses (itemize)			
Travel	750	Laundry	50
Slides & illustrations	200	Postage	50
Page charges and reprints	300		
Instrument repair & Maintenance	200		
Sub-Total for C			1,550
Running Total of A + B + C			$22,272

D. Permanent equipment (itemize)	
Exposure chambers	3,000
3 differential transducers	1,000
Pneumotachograph	200
Sub-Total for D	4,200
E	3,341
Total request	$29,813

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$17,118	5,000	1,500		3,543	27,161
Year 3						

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Respiratory tract retention of vapor phase constituents of cagarette smoke	AMA-ERF	$67,068	November 1, 1972 October 31, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name John L. Egle, Jr.

Signature John L. Egle, Jr. Date 1-24-75

Telephone 804 (Area Code) 770-4698 (Number) (Extension)

Checks payable to

Raymond T. Holmes, Jr.

Mailing address for checks

Mr. Raymond T. Holmes, Jr., Vice-Pres. Finance
1200 East Broad Street, Richmond, VA 23298

Responsible officer of institution

Typed Name Dr. John J. Salley

Title Asso. V-P for Research & Grad. Affairs

Signature John J. Salley Date 27 Jan 75

Telephone 804 (Area Code) 770-5608 (Number) (Extension)

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

January 31, 1975

Grant Application No. 1021

PULMONARY

To: The committee comprising Drs. Jacobson, Liebow and Sommers

Subject: Giles F. Filley, M.D., Webb-Waring Lung Institute, Denver
New application No. 1021
"Human Pulmonary Surfactant Function in Situ"

History

Application No. 1021 was handled by the Executive Committee as Case No. 300.

Request

This application requests $43,522 for the first year of a three year program.

Documents submitted (attached)

1. Application dated January 27, 1975 (25 pages, including C.V.'s of Drs. Filley, Paul and Newman, and G. Wayne Silvers).

2. Human subject consent form.

D.S.

DS:wg
Encl.

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#1021

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1/27/75

1. Principal Investigator (give title and degrees):

Giles F. Filley, M.D.
Professor of Medicine

2. Institution & address:

Webb-Waring Lung Institute
University of Colorado Medical Center
4200 East 9th Avenue
Denver, Colorado 80220

3. Department(s) where research will be done or collaboration provided:

Department of Medicine
and
Webb-Waring Lung Institute

4. Short title of study:

Human Pulmonary Surfactant Function In Situ

5. Proposed starting date: 7/1/75

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

These will be achieved by applying three disciplines (pathology, physiology and physical chemistry) to surfactant in situ.

1) To determine the post-mortem distribution of the alveolar lining layer in healthy and diseased lungs of cigarette smokers using special fixation methods.

2) To determine the functions of surfactant in these lungs by postmortem physiological studies of airway closure, acinar clearance and alveolar collapse.

3) To determine the physical properties (especially wettability and adhesiveness) of pulmonary surfactant on dissected lung tissues in comparison to the properties on synthetic substrates.

7a. Background information relevant to the objectives and working hypothesis:

The fact that surface phenomena contribute to lung retractive force, though reported by von Neergaard in 1929, was not generally appreciated till the direct demonstration of Pattle (1955) that the material lining alveoli had special properties. By squeezing bubbles from slices of lung at postmortem he obtained enough material to deduce one of its important physical properties--that its surface tension is "nearly zero" in vitro, that is to say, as the lining of a

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bubble outside the lung. His method of sampling the alveolar lining layer (as he called it) has been subsequently replaced in most laboratories by lung mincing or lung lavage methods, and the material so obtained has been subjected to extensive chemical analyses and surface balance studies. Valuable as these in vitro studies are, their indirect nature must be recognized and they must be correlated with the behavior of the lining layer in situ.

Because of the delicacy and inaccessibility of alveoli in the living lung very few direct physiological measurements have been made of alveolar wall deformation. In vivo observation of the lung surface (Wagner 1970) has not yielded quantitative information as to how this surface changes geometrically and our knowledge is largely limited to the results of measurements on quick frozen lung (Klingele and Staub 1970, Glazier 1967, Hughes et al 1970) or specially fixed tissue (Weibel and co-workers 1968, 1972, 1973). The latter have shown that alveoli are more polyhedral in shape than spherical, and that, at least in rat lungs fixed at three levels of inflation, the alveoli, rather than stretching with inflation, expand in a more complex manner perhaps best described as an "unpleating." The surface lining layer furthermore, rather than being a monolayer on a smooth surface, is distributed as sheets of variable thickness smoothing out tissue irregularities and as "pools of lining layer in crevasses." These authors have confirmed the finding of Klingele and Staub (1970) that below the physiologic range the alveoli "fold up from side to side as an accordion or concertina folds rather than by uniform decrease in all directions until they disappear as implicit in lung models based on the soap bubble analogy." Finally they have shown again in rat lungs, that the mean radius of curvature of expanded alveoli is about twice as large as in contracted alveoli and that the surface area-to-volume ratio was nearly constant instead of being inversely proportional to the radius of curvature as required by a spherical alveolar model.

How small airways 0.3 to 2.0 mm in diameter (alveolar ducts to 9th order bronchi) become narrowed and "close" is of vital importance to pulmonary physiology and chest medicine. Because of their location most investigations of small airway function have been indirect and their behavior has only recently begun to be understood (Macklem 1972). Direct intraluminal pressure measurements via tiny catheters have, thru the work of Macklem and his group (1965, 1967), revolutionized modern ideas of the mechanisms of airway obstruction and radically changed the meaning of standard ventilatory function tests (Mead 1970).

Excised lung tissue from experimental animals, meticulously dissected and studied by physiologic methods (Murtagh et al 1971, Menkes et al 1971, Hughes et al 1972), are being used to determine the relative contributions of intrinsic forces in airway walls, the luminal surface tension and the tissue forces of surrounding lung parenchyma. The recent review of Mead (1973) indicates the importance of these direct approaches to small airway function.

A recent direct attack on the problem of airway closure has been interpreted by the traditional concepts of how pulmonary surface forces operate, which we have examined above. Thus Macklem, Proctor and Hogg (1970) after recording

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P-V curves of air-filled dissected cat bronchioles before and after flushing with 2% Tween or dog lung extracts concluded "that surfactant lines and stabilizes bronchiles protecting against excessive radius changes with lung volume and air trapping. Their studies contradicted in some ways the finding of Cavagna et al (1967) that all airways are open at a transpulmonary pressure of zero, but the discrepancies were accounted for by "geometric factors" associated with distortion of airways consequent to the dissection procedure. The discrepancy between the results and those of others has been repeatedly referred to (Burger and Macklem 1968, Macklem et al 1970, Macklem 1971, Ingram et al 1974) and must be resolved.

Small airways, however, have many potential functions other than "staying open." A small airway's ability to convey liquid material originating from alveoli and, in particular to act as a conduit for the removal of toxic material from alveolar walls may be equally important. Unfortunately, although we know a fair amount about how ciliated airways are kept clear of unwanted material (Litt 1974), it is much less certain how alveolar tissue and small airway clearance mechanisms operate (Gross 1964, Staub 1966, Mendenhall 1972, Tucker et al 1973). New concepts are being developed to understand how cilia-free alveolar ducts and respiratory bronchiles clear themselves of noxious material. The importance of knowing whether or not surface active material is normally present and how it functions in the small airway lies in the possibility that changes in such material could well be produced by the inhalation of toxic substances. Thus DuBois and Rogers (1968) have concluded that the smaller bronchi are the most vulnerable sites for damage from inhaled particles. If surfactant is present in small bronchi, it can be shown (Sec. 9F) that toxic materials would be propelled toward the mouth by surface tension forces (i.e., without cough or ciliary action). If surfactant is absent or defective in the small airways of patients with emphysema, an obvious patho-genetic argument for the development of alveolar lesions early in this disease could be developed. Kilburn (1974) has presented evidence that certain small airways (those just at the junction of true alveolar tissue and the conduit bronchioles cleared by ciliary mechanisms), are a "no-man's land" as regards clearance, i.e. that they are in a region "cleared either way." It is clearly important to determine whether or not surface active material is present in such small airways and if it is capable of providing a clearance mechanism.

Finally, R.V. Ebert and M.J. Terracio have very recently (1975) shown that fresh surgical lung specimens from smokers show a loss of clara cells which may secrete a special sol (a strongly surface active colloid) in which cilia beat to move the overlying mucous gel. As Ebert says: "Unfortunately there is little direct evidence as to the nature of the surface coating of the bronchioles." Furthermore he emphasizes that when fixation via bronchi is used "the surface material is readily removed"--a point we consider in detail below (9.D.) The next page shows the delicacy of the surface we are considering as reproduced from page 9 of Ebert's paper.

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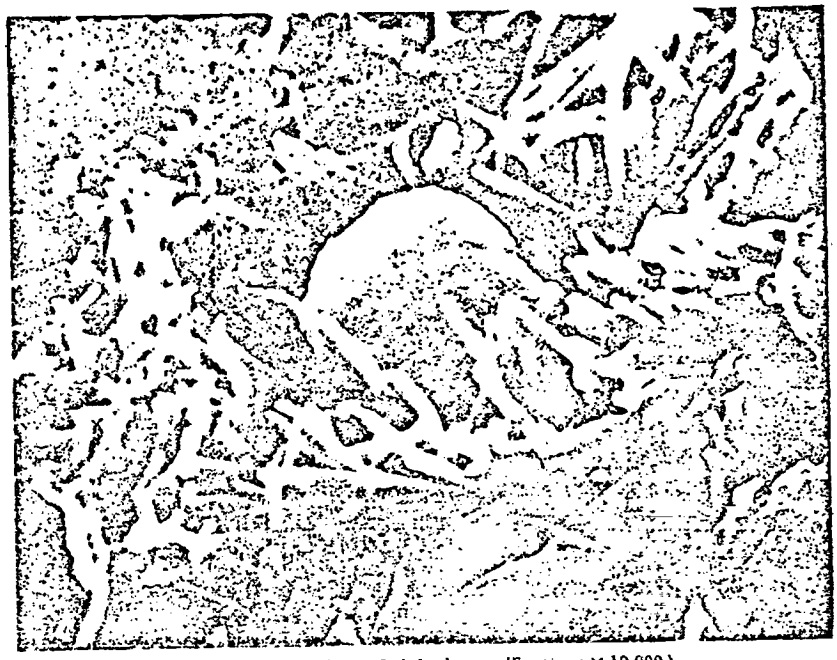

Fig. 5. A Clara cell surrounded by cilia. (Original magnification: × 10,000.)

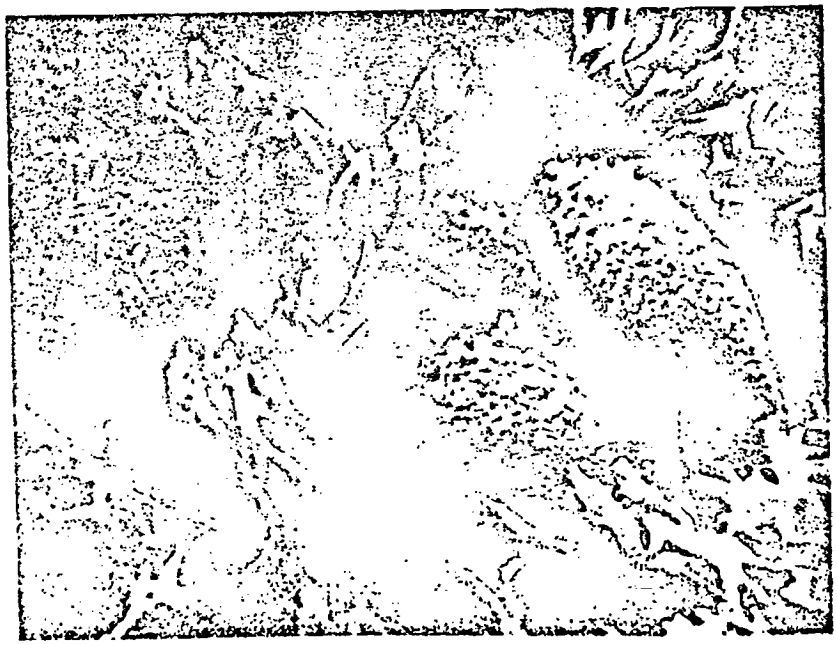

Fig. 6. Epithelium of a bronchiole 225 μm in diameter demonstrating transitional cells and ciliated cells. (Original magnification: × 5,250.)

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8. Brief statement of working hypothesis:

Although pulmonary surfactant is important in expanding the lungs of the newborn, its role in adult lungs, where it is continually produced is not understood. Despite the widespread belief that surfactant, by varying the air-alveolar surface tension, prevents alveoli from collapsing on expiration and from overstretching on inspiration, atelectatic or emphysematous adult human lungs have not been shown to yield abnormal surfactant on pulmonary lavage or by analysis of lung extracts. Our hypothesis is that surfactant provides an important protective function and that maldistribution of surfactant allows cigarette smoke to narrow airways, promote alveolar pleating and damage Type I alveolar cells.

Failure of surfactant to form a ubiquitous layer and convey deposited material from the acinus would expose Type I cell walls to direct and prolonged effects of inhaled substances. The remarkable fact that the vast majority of cigarette smokers do not acquire emphysema suggests that most human lungs are as efficient in protecting their alveoli as tears are in protecting the eyes. To study the chemistry of tears in vitro would reveal very little of their protective function. Thus our insistence that bronchial and alveolar surfaces be studied in situ.

9. Details of experimental design and procedures (append extra pages as necessary)

A. Sources of human lungs, temporal effects and pathology

Human lungs will be obtained from the autopsy service of the Department of Pathology of the University of Colorado Medical Center. A special source will be cadaver transplant donors (kidney and liver) whose usually normal lungs will be made available by the Department of Medicine (Dr. T.L. Petty) and the Division of Biochemistry of the Webb-Waring Lung Institute (Dr. O.K. Reiss).

There are two potential sources of error which must be considered in regard to studies of pulmonary surfactant when the source of the lungs is human beings at autopsy, namely, the time postmortem and the method of sampling surface-active lung fluids. Avery and Mead (1959) reported that in dog lungs there was no significant difference in their surfactant assay results if lungs were studied immediately after death or after refrigeration or freezing for as long as six days. Gruenwald et al (1962) showed that adult human lungs frozen at -20° C and examined six weeks after autopsy were considered satisfactory. Reynolds et al (1965) demonstrated that in lungs of newborn infants obtained within 24 hours of death and stored at -4° to -10° C and then studied within three days, there was no change when surfactant assay was re-examined 12 months after storage. However, the effect of time postmortem on pressure-volume measurements is still not settled. Faridy et al (1966) have shown that dog lobes do not show significant differences in pressure-volume measurements if the lobes were at low temperature for five days, yet Bachofen et al (1970) showed slight changes in pressure-volume curves of cat lungs after three hours after death. Accordingly, we will have to determine the time postmortem effects of the human lungs with which we will be working. We will do this by carrying out repeated pressure-volume and surfactant assay measurements.

Facilities of the Division of Pathology of this Institute will be used to assess the lungs for their normality and to determine the nature of any pathological processes in the tissue. Smoking histories will be documented when available.

B. Surfactant isolation

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For the experiments in Sec. 9G, we will isolate surfactant material by saline lavage (0.9% NaCl) of human postmortem lungs. Serial lavages will be pooled and

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centrifuged at 850 xg to remove alveolar macrophages and other cellular debris. The supernatant will be spun at 10,000 xg to concentrate the surface active material into a pellet. The pellet, which will be resuspended in 0.9% NaCl, is referred to as whole or crude surfactant preparation.

The isolation procedure proposed here is based on experience in this laboratory with rabbit, pig and sheep lung surfactant. Each step will therefore have to be checked for its applicability to the isolation of human material, and this is currently under way in the laboratory of Dr. O.K. Reiss at this Institute. When it becomes desirable to study the purified lipid and protein fractions, such material will be obtained by the zonal density centrifugation methods worked out in this laboratory (Reiss 1970, Gil and Reiss 1973).

The use of micropipettes for direct sampling of surfactant from the alveolar surface and micromethods for the study of its physicochemical properties has been reported by Reifenrath and Zimmermann (1974). Since direct methods have proven decisive in other areas of this field these micromethods seem worth exploring. Staub (1974) has reported the astounding fact that in a related field of tremendous clinical importance (pulmonary edema physiology) "No one except Nitta (1973) has ever obtained direct alveolar fluid." Direct microsampling methods of surfactant isolation will be used as micromethods of physical and chemical study develop (second and third year).

C. In situ P-V methods

Pressure-volume measurements

Whole human lungs will be ventilated with air and with liquids to determine their quasi-static P-V characteristics; in addition, lobes, segments and sub-segments will be dissected from whole lungs and similarly ventilated. Lungs and portions of lungs will be placed in a volume-displacement plethysmograph similar to the one described by Bachofen et al (1970). This apparatus allows air and liquid pressure-volume curves as well as degassing to be performed without removing the tissue from the plethysmograph.

Our experience with degassing human lungs in the manner often done with animal lungs (Johnson et al 1964), by placing them in a vacuum jar and evacuating the jar until water vaporization and recompression, often resulted in tears and rupture of the parenchyma. This we felt was due to collapse of the small airways trapping gas in the alveoli and eventual rupture of the parenchyma on further evacuation of the vacuum jar. This is overcome by the technique of von Neergaard (1929). A water valve set at 8-10 cm H_2O is inserted between the cannula to the lung and the plethysmograph. With the evacuation pump operating simultaneously both on the bronchus and the space between the lung and plethysmograph, the pressure outside the lung is maintained to about 8-10 cm water lower than in the bronchus. The alveoli thus are in free communication with the bronchial tree until full evacuation, i.e., water vapor pressure, is reached. This will allow filling with air or liquid without any walls being opposed or "stuck together," which filling may well give different results than when collapse is allowed to occur as in conventional degassing. The two techniques will be compared.

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An air pressure-volume curve will be done after degassing. The lungs will be inflated to +20 to 22 cm water and deflated to zero pressure three times to standardize volume history. Beginning with the fourth inflation, volume will be

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monitored at 1 cm of water pressure increments until +20 cm of water is reached. Thirty seconds will be allowed between measurements for equilibration of pressure. The same procedure will be followed on the deflation limb of the pressure-volume curve.

As indicated above we will dissect, from normal and pathologic human lungs, portions for direct study of mechanical and other properties connected with surface activity. Isolation of the superior segment of the right lower lobe has already been accomplished (see Fig. 1) along with cannulation of the superior segmental bronchus, B_6, (Bloomer et al 1960). The problem of leaks has been dealt with by gluing the edges of the cut surface to a glass plate with contact cement (Permabond 102, Pearl Chemical Co., Tokyo). Dissection of subsegments of the superior segments of both the right lower and left lower lobes will be carried out with the help of a Magnifying Loupe, Iris forceps, Micro Jameson Scissors and similar surgical equipment (already purchased on the advice of Dr. Melvin Newman). By making use of many representative portions of human lungs we hope to obtain the maximum information possible from a single human lung, especially when the experiments involve ventilation and fixation at various levels of inflation with expensive materials.

D. In situ surfactant distribution

The very recent report of Callas (1974) shows that even after the painstaking work of Gil and Weibel (1969-70) "all published reports indicate failure to demonstrate surfactant as a layer lining the entire alveolar surface." Callas has modified Weibel and Gil's method by attempting to "plaster" the surfactant to the alveolar cells by filling the lungs with agar. In doing this, however, he is compelled to force the agar into a completely collapsed lung (since the air in an inflated lung would prevent the agar from penetrating to alveolar surfaces). Despite the use of his elaborate method, Callas makes the remarkable statement that "no alveoli were found to be completely lined with surfactant."

We believe that no method which introduces a liquid into a lung (especially one that is collapsed) can reveal the normal in situ distribution of surfactant. We therefore propose to use the special degassing technique worked out by von Neergaard (see Sec. 9C) for another purpose. The lungs, instead of being degassed in the usual way in which alveolar surfaces are allowed to fold up, collapse and touch each other (which, we think, must considerably change the distribution of surfactant), are degassed by removal of air molecules without change of lung volume. After this has been accomplished fixation by vaporized or aerosolized glutaraldehyde will be carried out. This method will imitate, to some extent, the formalin steam method of Weibel and Vidone (1961), but will be modified to insure that the lung neither gains nor loses water (Wright et al 1974). In this way the alveolar lining layer will be fixed in situ, its distribution unaffected by processing and fixation artefacts--probably for the first time.

After glutaraldehyde fixation both conventional and electron microscopy methods will be used. Briefly, we will use the ultravioled microscopy technique of Balande and Klause (1964), who concluded that the alveolar lining layer was revealed in guinea pig lungs by fluorescent lines less than one micron thick. The alveolar lining layer of human infants has also been observed using this technique (DeSa 1965). The same tissue fixed in the previously described manner

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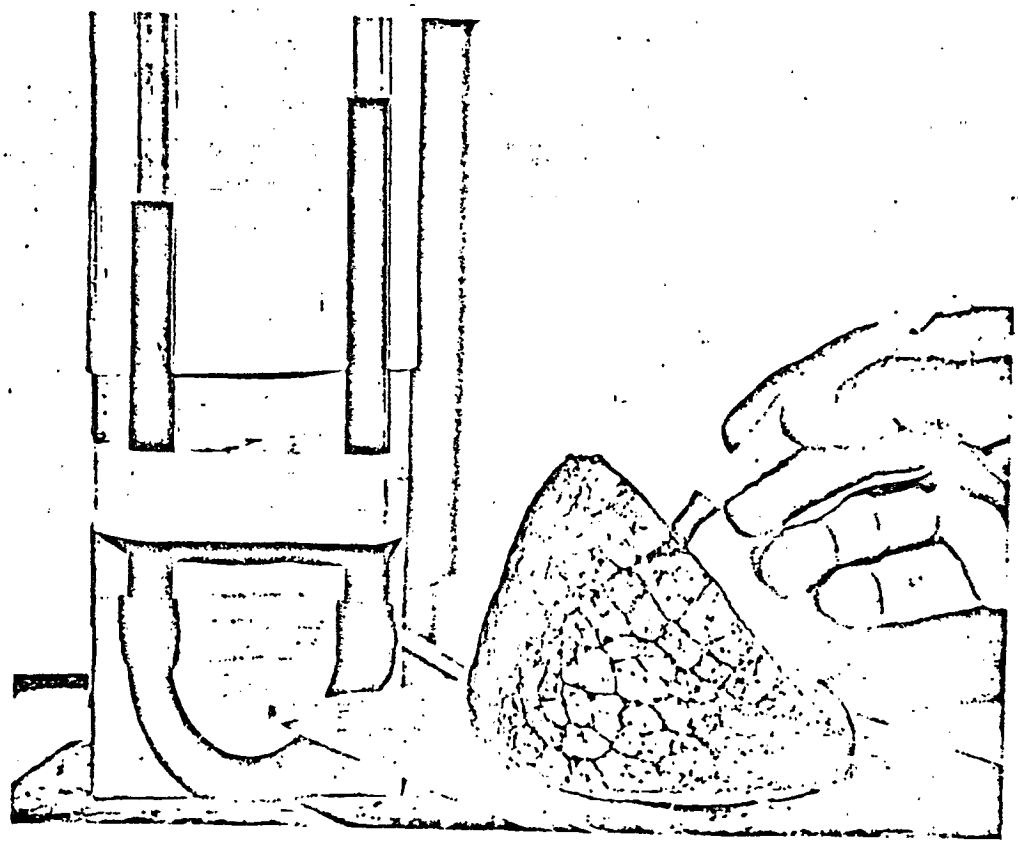

Fig. 1. Superior segment of the right lower lobe of a normal human lung. The cut surface of the posterior basal lobe is resting on a glass plate and glued at the edges. The superior segmental bronchus is cannulated for P-V measurements.

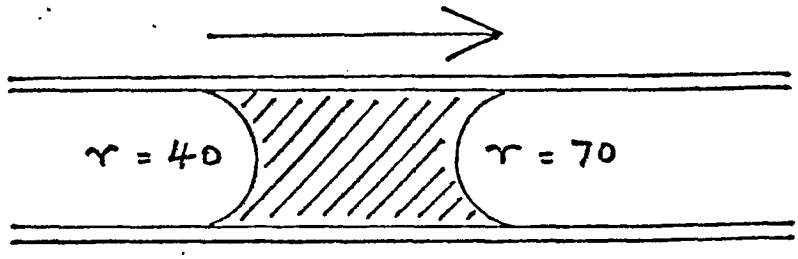

Fig. 2. The fluid is "pulled" in the direction of the arrow by the greater surface energy at the right meniscus. If the right meniscus is facing the trachea or the left the alveoli, surface energies could have a definite role in clearing material from small airways (Schwartz et al., 1964).

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may be used for electron microscopy. Post-fixation methods as described by Callas (1974) will be used in conjunction with the EM facilities of the Division of Cell Biology of this Institute.

E. Methods for the direct study of airway closure mechanisms in the lungs of smokers and non-smokers

Lungs which have had pressure volume curves constructed, as described above, will be dissected to remove a section of the bronchial airways of 2-3 mm in diameter and less. (Surgical advice and assistance will be given to us by Dr. Melvin Newman.) The largest airway will be cannulated while the rest will be tied off with suture. The specimen will be placed in a small plethysmograph equipped with an optical window for microscopic observation similar to that described by Martin and Proctor (1958). The cannulae will be 10 and 20 microliter pipettes, which will be calibrated previously with a Hamilton microliter syringe. A small droplet of water will be placed within the pipette and will serve to mark the volume of air entering the airways. Pressure will be measured by use of a leveling bulb, on a Statham strain-gauge pressure transducer (P23Gb). Thirty second intervals will be allowed for equilibration. A minimum of four complete inflation and deflation pressure-volume curves will be done to check on reproducibility. A similar procedure will be followed when pressure-volume curves of the specimen in various liquids are to be obtained. The plethysmograph will be completely filled with liquid as well as the leveling bulb so that now pressure readings will be obtained by reading the difference between the menisci of the pipette and leveling bulb. The pressures at which the airways open and close will be recorded. From these data we can determine if the airways close at the same or at different pressures in the air filled lung, indicating if surface forces influence small airway closure, and relating these findings to the smoking habits of the patient in life.

Direct microscopy will also be used to determine the relative roles of surface intrinsic and extrinsic forces controlling the behavior of the 0.3-2.0 mm airways of human lobes. Lobes of lungs obtained at surgery or autopsy will have the tracheobronchial tree exposed by dissection with the aid of a dissecting microscope. By cannulating the airways with a blunt needle, pressure-volume curves can be achieved with a microliter syringe and a small volume displacement pressure transducer as described by Macklem et al (1970), the pressures at which different sized airways open and close can be measured and noted with the dissecting microscope. This procedure can be performed by using air, saline, or by flushing with a substance with a known surface tension such as Tween, reflushing with air and repeating the measurements using air. In this manner we can change the surface tension from that normally found in airways to that approaching the tension of the fluid just flushed. Because the geometry and mechanics of dissected airways differs from that of bronchi in the intact lung, we will, in the above methods, be relying on the comparative behavior of airways lined with different materials. If initial volumes and pressures are held constant for each experiment, the differences between P-V relationships should be informative even though the "extrinsic factors" due to lung parenchyma are different than in life (Murtagh et al 1971, Hughes et al 1972, Hughes et al 1973).

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To determine the geometry of closure of airways (lined with naturally occurring surfactant or other materials) as lung tissue volume is reduced, the quantitative methods of Gil (1971) will be used. The bronchi and pulmonary artery supplying the

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lobes or segments of human lungs will be cannulated. By controlling the airway pressure, the lung volume and the flow of fixative, the lungs will be fixed at several points along both inflation and deflation limbs of the P-V curve. Fixation of lungs and appropriate sectioning and microscopic study of the small airways will reveal the morphologic changes they undergo as they "close" during deflation and "open" from a state of closure and should clarify the nature of airway P-V hysteresis (Mead 1973).

To localize portions of airways of particular interest we will, before fixation and/or sectioning, use the facilities of the Magnification Unit in the Department of Radiology. Here a FAXITRON X-ray apparatus (Hewlett-Packard) will enable us to use low energy X-rays. The device has an ultrafine focal spot (0.5 mm) and a beryllium window. Thin pieces of lung will be examined in order to minimize distortion from overlapping of airways. The advantage of this technique is that it allows us to visualize airways as small as 200 μ in diameter and to avoid the use of contrast media after the manner of Murtagh et al (1971).

F. Method for the direct study of airway clearance mechanisms

As Macklem has postulated (1971), based on direct observation of dissected bronchioles, fluid in small airways could form two hemispherical menisci in vivo, one facing the trachea and the other facing the alveoli. His analysis was restricted to considerations of airway stability. However, such fluid menisci are known to have properties which affect fluid movement and which may be important as part of a lung clearance mechanism.

Well-developed formulations (Davies and Rideal 1961, Schwartz et al 1964) relate the movement of a liquid through air-filled horizontal tubes to the surface tension of the liquid and the contact angle between the liquid and tube wall surface. Both theoretically and experimentally, liquid segments of the type visualized by Macklem move through capillary tubes in directions and at rates determined by the difference between the surface tensions at the two menisci. (See Fig. 2). Using the methods discussed under Sec. 9E for dissecting out and studying the bronchi of human lungs, we will investigate, by direct microscopic observation, the effect of naturally present and foreign surface-active materials to the importance of locally varying surface tensions in clearing material from alveolar tissue.

G. Wettability and adhesive properties of pulmonary surfactant on dissected lung tissues and on synthetic materials

As a result of our independent theoretical development, we have concluded that there are several "surface tensions" of importance in the lung. We visualize the surfactant system of the lung as a lining layer (ll) of surfactant "sandwiched" between the air (a) phase and the tissue hypophase (h). The tensions (γ) between the lining layer and air or tissue are given by $\gamma_{ll,a}$ and $\gamma_{h,ll}$ respectively; at places where the tissue is not covered with surfactant the tension is $\gamma_{h,a}$. The relationship among the tensions is given by the equation of Young (1805):

$$\gamma_{h,a} - \gamma_{h,ll} = \gamma_{ll,a} \cos\theta$$

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where θ is the contact angle between the surfactant layer and the tissue. $\theta = 0$ is the condition for complete spreading of surfactant on the tissue.

The purpose of the wetting experiments is to infer the contribution of the tensions at the tissue-air and tissue-surfactant interfaces to the total surface force in the lung, and to determine the spreading characteristics of surfactant on normal and diseased tissue. This is important because, for example, cigarette smoke may alter the tissue while the surfactant appears "normal."

Thin slices of alveolar tissue or small airways, the cut surfaces on which the wettability and adhesion of surfactant will be measured, will be obtained with a tissue slicer or by hand dissection. Chemical fixatives will of course have to be avoided because of possible alteration of the surface properties of the tissue. For the present experiments we will use an automated Wilhelmy film balance of conventional design. Details of construction for the particular balance to be employed have been previously described (Dreher and Wilson 1970). The balance has since been modified to measure γ-A isotherms over a wide range of frequency and to interface with a H-P 7001 x-y recorder. The balance will be suitably mounted to eliminate the effect of vibrations, and cleanliness of chemicals, glassware and surfaces will be in accordance with accepted procedures (Gaines 1966).

Young's equation may be rewritten as $\gamma_{adh} = \gamma_{11,a} \cos\theta$, where $\gamma_{adh} = \gamma_{h,a} - \gamma_{h,11}$ is the adhesion tension. The completeness and reversibility of spreading will be determined by the wetting balance method of Guastalla (1957). If $\theta \neq 0$ then a Wilhelmy plate of platinum measures $\gamma_{11,a} \cos\theta$. Thus, if a plate is constructed of a thin slice of lung tissue (or two slices of tissue with a thin piece of glass or metal sandwiched between them for stability) and the force $\gamma_{11,a}\cos\theta$ transmitted to this tissue plate by a spread film of surfactant on water is measured, then the simultaneous measurement of $\gamma_{11,a}$ with a roughened ($\cos\theta=1$) platinum or glass plate in another part of the film will yield the value of $\cos\theta$ as well as γ_{adh}. In this way the validity of the assumption that surface forces at the air-water interface are similar to the forces on the surface of the lung can be tested in a more direct manner than has heretofore been proposed. For example, if the advancing and receding contact angles are widely different, the γ-Area hysteresis loop traced by the force on the tissue plate would reflect the importance of wettability to the P-V loops in air filled lungs. Similarly, by substituting a liquid for air, the γ-Area loops so obtained should reflect conditions at the interface of a liquid filled lung.

To estimate $\gamma_{h,a}$, the $\cos\theta$ of droplets of low energy liquids (fluorocarbons) of known surface tension ($\gamma_{f,a}$) will be measured on lung tissue which has been rinsed with saline. $\gamma_{h,a}$ may be estimated by application of the equation of Good (1964) and Fowkes (1962):

$$\gamma_{h,a} = \gamma_{f,a} (1 + \cos\theta)^2 / 4\phi^2 \gamma_{f,a}$$

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where ϕ is a numerical constant. Lung tissue strips will be mounted horizontally in a small plexiglas chamber with ports for evacuation or saturation with gas. The θ's will be measured with a cathetometer telescope and a gionometer eyepiece. The magnitude of $\gamma_{h,a}$ and $\gamma_{h,11}$ (which is now known from $\gamma_{h,a}$ and the previously measured γ_{adh}) relative to $\gamma_{11,a}$ will reflect the importance of the tissue stress in the total retractive force in the lung.

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Methods of measuring the adhesive force between similar or dissimilar surfaces coated with a liquid film have been known for many years (Budgett 1912). These forces are often quite high, thus it is worthwhile to determine if reductions in the adhesive force can be effected by pulmonary surfactant. This problem will be approached by using a duNouy tensiometer to measure the vertical force required to separate plates of synthetic materials (glass, metal, teflon) or slices of lung tissue coated with surfactant. Since lung surfactant is known to be viscoelastic (Kott et al 1974), the results will depend on the rate of separation of the surfaces. Initially the experiments will be confined to quasi-static conditions. By comparing the work required to separate lung tissue in air with that required in saline, the idea is to show that the known reduction in retractive force in liquid filled lungs relative to air filled lungs can be explained by an alternate mechanism of surfactant operation. For example differences in the air and saline work of adhesion would indicate that surfactant could produce the difference seen between air and liquid P-V curves without significant changes in lung area by stretching of the alveolar surface, but rather by a simple unfolding of alveolar pleats.

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References

Avery ME, Mead J: Surface properties in relation to atelectasis and hyaline membrane disease. J Dis Children 97: 517, 1959.

Bachofen H, Hildebrandt J, Bachofen M: Pressure-volume curves of air- and liquid-filled lungs--surface tension in situ. J Appl Physiol 29: 422, 1970.

Balande RP, Klaus MH: The morphologic demonstration of an alveolar lining layer and its relationship to pulmonary surfactant. Am J Pathol 45: 449, 1964.

Bloomer WE, Liebow AA, Hales MR: Surgical anatomy of the bronchovascular segments. C.C. Thomas, Springfield, Ill., p. 99, 1960.

Budgett AM: Proc Roy Soc (Lon) Ser A. 86: 25, 1912.

Burger EG Jr, Macklem PT: Airway closure demonstration by breathing 100% O_2 at low lung volumes and by N_2 washout. J Appl Physiol 25: 139, 1968.

Callas G: A new fixation technique for the electron microscopic study of pulmonary surfactant. Anat Rev 180: 457, 1974.

Cavagna GA, Stemmler EG, DuBois AB: Alveolar resistance to atelectasis. J Appl Physiol 22: 441, 1967.

Davies JT, Rideal EK: Interfacial Phenomena. Academic Press, New York, 1961.

DeSa DJ: Microscopy of the alveolar lining layer in newborn infants. Lancet 1: 1369, 1965.

Dreher KD, Wilson JE: Structure and specific counter ion effects of nonadecylbenzenesulfonate monolayers. J Coll Interface Sci 32: 248, 1970.

DuBois AB, Rogers RM: Respiratory factors determining the tissue concentration of inhaled toxic substances. Resp Physiol 5: 34, 1968.

Ebert
see p2k
Faridy EE, Permutt S, Riley RL: Effect of ventilation on surface forces in excised dogs' lungs. J Appl Physiol 21: 1453, 1966.

Fowkes FM: Determination of interfacial tensions, contact angles and dispersion forces in surfaces by assuming additivity of intermolecular interactions in surfaces. J Phys Chem 66: 382, 1962.

Gaines GL Jr: Insoluble monolayers at liquid-gas interfaces. Interscience Publishers, New York, 1966.

Gil J: Ultrastructure of lung fixed under physiological conditions. Arch Int Med 127: 896, 1971.

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Gil J, Weibel ER: Improvements in demonstration of lining layer of lung alveoli by electron microscopy. Resp Physiol 8: 13, 1969-70.

Gil J, Weibel ER: Morphological study of pressure-volume hysteresis in rat lungs fixed by vascular perfusion. Respir Physiol 15: 190, 1972.

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Gil J, Reiss OK: Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. J Cell Biol 58: 152, 1973.

Glazier JG, Hughes JMB, Maloney JC, West JB: Vertical gradient of alveolar size in lungs of dogs frozen intact. J Appl Physiol 23: 694, 1967.

Good RJ: Theory for the estimation of surface and interfacial energies. Contact Angle, Wettability and Adhesion. Adv Chem Ser 43, ACS Publications, Washington, D.C. 74, 1964.

Gross P: The processes involved in the biologic aspects of pulmonary deposition, clearance and retention of insoluble aerosols. Health Phys 10: 995, 1964.

Gruenwald P, Johnson RP, Hustead RF, Clements JA: Correlation of mechanical properties of infant lungs with surface activity of extracts. Proc Soc Exptl Biol Med 109: 369, 1962.

Guastalla J: Proc 2nd Intl Congr Surface Activity. Butterworths, London 3: 143, 1957.

Hughes JMB, Rosenzweig DY, Kivitz PB: Site of airway closure in excised dog lungs: Histologic demonstration. J Appl Physiol 29: 340, 1970.

Hughes JMB, Hoppin FG, Mead J: Effect of lung inflation on bronchial length and diameter in excised lungs. J Appl Physiol 32: 25, 1972.

Hughes JMB, Hazel AJ, Wilson AG: Extrinsic and intrinsic hysteresis of intra-pulmonary bronchi in situ. Proc Physiol Soc 234: 92P, 1973.

Ingram RH Jr, O'Cain CF, Fridy WW Jr: Simultaneous quasi-static lung pressure-volume curves and "closing volume" measurements. J Appl yhysiol 36: 135, 1974.

Johnson JWC, Permutt S, Sipple JH, Salem ES: Effect of intra-alveolar fluid on pulmonary surface tension properties. J Appl Physiol 19: 369, 1964.

Kilburn KH: Clearance zones in the distal lung. Ann NY Acad Sci 221: 276, 1974.

Klingele TG, Staub NC: Alveolar shape chagnes with volume in isolated, air-filled lobes of cat lung. J Appl Physiol 28: 411, 1970.

Kott AT, Gardner JW, Schecter RS: The elasticity of pulmonary lung surfactant. J Coll Interface Sci 47: 265, 1974.

Litt M: Rheological aspects of microciliary flow. Ann NY Acad Sci 221: 212, 1974.

Macklem PT, Wilson NJ: Measurement of intrabronchial pressure in man. J Appl Physiol 20: 653, 1965.

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Macklem PT, Mead J: Resistance of central and peripheral airways measured by a retrograde catheter. J Appl Physiol 22: 395, 1967.

Macklem PT, Proctor DF, Hogg JC: The stability of peripheral airways. Respir Physiol 8: 191, 1970.

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Macklem PT: Airways obstruction and collateral ventilation. Physiol Rev 51: 368, 1971.

Macklem PT: Obstruction in small airways--a challenge to medicine. Am J Med 52: 721, 1972.

Martin HB, Proctor DF: Pressure-volume measurements on dog bronchi. J Appl Physiol 13: 337, 1958.

Mead J: The lungs' "quiet zone". Editorial, New Engl J Med 282: 1318, 1970.

Mead J: Respiration: pulmonary mechanics. Ann Rev Physiol 35: 169, 1973.

Mendenhall RM: Surface spreading of lung alveolar surfactant. Resp Physiol 16: 175, 1972.

Menkes A, Gardiner A, Gamsu G, Lampert J, Macklem PT: Influence of surface forces on collateral ventilation. J Appl Physiol 31: 544, 1971.

Murtagh PS, Proctor DF, Permutt S, Kelly BL, Evering ES: Bronchial mechanics in excised dog lobes. J Appl Physiol 31: 403, 1971.

Nitta S, Staub NC: Lung fluids in acute ammonium chloride toxicity in cats and guinea pigs. Am J Physiol 224: 613, 1974.

Pattle RE: Surface lining of lung alveoli. Physiol Rev 45: 48, 1965.

Reifenrath R, Zimmermann I: Surface tension properties of lung alveolar surfactant obtained by alveolar micropuncture. Respir Physiol 19: 370, 1974.

Reiss OK: The purification and analysis of lipoproteins from lung. Abstr Am Chem Soc Meeting, 160th, Chicago, 1970.

Reynolds EDR, Orzalesi MM, Matoyama ED, Craig JM, Cook CD: Surface properties of saline extracts from lungs of newborn infants. Acta Paed Scand 54: 511, 1965.

Schwartz AM, Rader CA, Huey E: Resistance to flow in capillary systems of positive contact angle. Adv Chem Ser 43: ACS Publications, Washington DC, 250, 1964.

Slama H, Schoedel W, Hansen E: Lung surfactant: film kinetics at the surface of an air bubble during prolonged oscillation of its volume. Respir Physiol 19: 233, 1973.

Staub NC: The "liquid veins" of the lung. Abstr, Physiologist 9: 294, 1966.

Staub NC: Pulmonary edema. Physiol Rev 54: 678, 1974.

Tucker AD, Wyatt JH, Undery D: Clearance of inhaled particles from alveoli by normal interstitial drainage pathways. J Appl Physiol 35: 719, 1973.

von Neergaard K: Neue Auffasungen über einen Grundbegriff der Atemmechanik. Die Retraktionskraft der Lunge, Abhängig von der Oberflächenspannung in den Alveolen Z Ges Expl Med 66: 373, 1929.

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Wagner WW Jr: Pulmonary microcirculatory observations in vivo under physiological conditions. J Appl Physiol 26: 375, 1970.

Weibel ER, Vidone RA: Fixation of the lung by formalin steam in a controlled state of air inflation. Am Rev Resp Dis 84: 856, 1961.

Weibel ER, Gil J: Electron microscopic demonstration of an extracellular duplex lining layer of alveoli. Respir Physiol 4: 42, 1968.

Weibel ER: Morphological basis of alveolar capillary gas exchange. Physiol Rev 53: 419, 1973.

Weibel ER, Untersee P, Gil J, Zulauf M: Morphometric estimation of pulmonary diffusion capacity. VI. Effect of varying positive pressure inflation of air spaces. Respir Physiol 18: 285, 1973.

Williams R: Computer analysis and model of the properties of pulmonary surfactant: effects of nitrogen dioxide. Ph.D. Dissertation, Pennsylvania State Univ, University Microfilms, Ann Arbor, Mich, 1970.

Wright BM, Slavin G, Kreel L, Callan C, Sandin B: Postmortem inflation and fixation of human lungs. Thorax 29: 189, 1974.

Young T: On the cohesion of liquids. Phil Trans 95: 65, 82, 1805.

Ebert VR, Terracio MJ: The bronchiolar epithelium in cigarette smokers. Observations with the scanning electron microscope. Am Rev Resp Dis 111: 4, 1975.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

A 600-square foot laboratory in the basement of the Webb-Waring Lung Institute (W-WLI) is available for the postmortem lung mechanics studies and for the isolated surfactant studies. A pathology laboratory equipped for inflation-fixation and cutting of lungs, preparation of histologic sections and long-term storage of fixed lung slices and histologic material is available on the fourth floor of W-WLI.

Major items of equipment availabor for this work at W-WLI include:
Electronics for Medicine IR-r recording equipment
Hewlett-Packard 7001 x-y recorder
Beckman GC2-A gas chromatograph
Electronics for Medicine DR-8 and assorted transducers
Monoghan respirator
Harvard respiration pump
Beckman Spinco Model L Ultracentrifuge
International Refrigerated Centrifuge Model PR-2
Sorvall RC2-B refrigerated centrifuge
Mettler M-5 microbalance
Automatic quartz bi-distillation unit for production of pure water
Automated Wilhelmy surface balance modified for variable frequency and temperature controlled gas studies
Collins spirometer
Phillips 300 Electron Microscope

The Department of Radiology is equipped with Faxitron X-ray facilities and auxiliary equipment.

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

CURRICULUM VITAE

GILES F. FILLEY, M.D.

June 1972

Birth Date: REDACTED

Marital Status: REDACTED

Children: REDACTED

Education: Kent School, Kent Conn. Graduated R High School
Williams College, Williamstown, Mass. Graduated R A.B.
Johns Hopkins University, Baltimore, Md. Graduated R M.D.

Internship: Johns Hopkins Hospital, Baltimore, Md. 1942-1943.

Residencies: Lawrason Brown Fellow, Department of Physiology, The Edward L. Trudeau Foundation, Saranac Lake, New York 1943-1944.

Assistant in Medicine, Johns Hopkins University. 1944-1945.

Instructor in Medicine and John D. Archbold Fellow in Medicine, Johns Hopkins University. 1945-1946.

Assistant Resident Physician, Medical Ward Service, Barnes Hospital, St. Louis, Mo. 1946-1947.

Positions: Associate Physiologist, Department of Physiology, The Edward L. Trudeau Foundation, Saranac Lake, New York. 1947-1953.

Director, Department of Physiology, The Trudeau-Saranac Institute, Saranac Lake, New York. 1953-1955.

Assistant Professor of Medicine, University of Colorado School of Medicine, Denver, Colorado. 1955-1959.

Associate Professor of Medicine, University of Colorado School of Medicine, Denver, Colorado. 1959-1969.

Professor of Medicine, University of Colorado School of Medicine, Denver, Colorado. 1969-Present.

Clinical Physiologist, Chief-Division of Physiology, Webb-Waring Lung Institute, Denver, Colorado. 1955-Present.

Memberships in Societies: REDACTED

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Consulting Positions: Denver General Hospital
Fitzsimons Army Hospital
Veterans Administration Hospital

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R: REDACTED MATERIAL

RESUME

George W. Paul

REDACTED

(303) 394-8731 (Office)

PERSONAL

REDACTED REDACTED

RESEARCH INTERESTS

Biomedical applications of surface chemistry, structure of water and liquid solutions, membrane phenomena, thermodynamics (equilibrium and non-equilibrium), transport processes. Have reviewed about 400 papers in these areas. Long range: Teaching or research institute.

EDUCATION

University of Missouri (Columbia): REDACTED M.S. R
Ph.D. R all chemical engineering. REDACTED

M.S. Thesis: "Interfacial Free Energy in Binary and Ternary Systems."

Ph.D. Comprehensive Problem: "An Investigation of Solute Diffusion in Polymer Solutions."

Ph.D. Dissertation: "Interfacial Tension in the Critical Region."

EXPERIENCE

Summer 1963: U.S. Gypsum Co., Mexico, Missouri. Polymer coating technology.

Summer 1964 and 1965: Nalco Chemical Co., Chicago, Illinois. Correlation of kinetics of co-polymerization reactions.

Summer 1966 and 1967: Research Assistant, Engineering Experiment Station, University of Missouri, Columbia, Missouri. Undergraduate instruction in techniques of digital computation.

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8/69 to 11/72: Standard Oil Co. (Ind.) Research Center, Tulsa, Oklahoma. Surfactants and biopolymers (polysaccharides). Evaluation of experiments on micellization, non-equilibrium adsorption, flow through porous media.

11/72 to present: Postdoctoral Research Fellow, Webb-Waring Lung Institute, University of Colorado Medical Center, Denver, Colorado. My present work is focused on understanding the physical chemistry of the lining of human lungs. The surface of mammalian lungs in general is composed of a special fluid of peculiar surface properties. It is known that some diseases result directly from disturbances in these surface properties. It is likely that even more common diseases such as emphysema involve changes in the chemistry of these substances and my work is devoted to determining these changes.

ACTIVITIES

1968, Teen Club Worker, Columbia, Missouri. Avid tennis player, reader of contemporary history.

PROFESSIONAL ORGANIZATIONS

REDACTED

REDACTED REDACTED

HONORS AND AWARDS

Green Scholarship, Curators Scholarship, American Chemical Society Research Fellowship, NASA Fellowship, Omicron Delta Kappa, Pi Omicron Sigma.

REFERENCES

Professor Marc deChazal, Department of Chemical Engineering, University of Missouri, Columbia, Missouri 65201; Dr. J.T. Ryan, Department of Chemical Engineering, University of Alberta, Edmonton, Alberta, Canada; Dr. P.D. Shoemaker, Monsanto Company, 800 N. Lindbergh, St. Louis, Missouri.

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R: REDACTED MATERIAL

Resume

G. Wayne Silvers

Born: REDACTED

Education: University of Colorado, Boulder, Colorado
B.A., Zoology, R

Appointments: Research Associate, University of Colorado Medical Center, Denver, Colorado, 1972 - present.

Research Physiologist, University of Colorado Medical Center, 1967-1972

Research Technician, University of Colorado Medical Center, 1963-1967.

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CURRICULUM VITAE

Melvin M. Newman, M.D.
REDACTED

Place and Date of Birth:	
College:	University of Chicago, REDACTED
Medical School:	University of Chicago, R B. S. R M. D. R
Honor Societies:	Phi Beta Kappa, Alpha Omega Alpha, Sigma Xi
Rotating Internship:	University of Chicago Clinics, September 1944 - June 1945
Residency Training:	University of Chicago Clinics, October 1946 - July 1952 General and Thoracic Surgery
Teaching and Research Appointments:	Instructor in Surgery, University of Chicago, July 1950-- June 1952
	National Research Council Fellow, University of Chicago July - October 1952 (Recalled to Active Duty, U.S. Navy)
	Assistant Professor of Surgery, State University of New York College of Medicine at Brooklyn, July 1954 - March 1956
	Associate Professor, March 1956 - August 1959
	Director of Surgery, National Jewish Hospital at Denver, September 1959 to August 1968
	Clinical Associate Professor of Surgery, University of Colorado, 1961 to 1968
	Associate Professor of Surgery (Full-time), University of Colorado, 1968 to present
Certification:	American Board of Surgery - 1951; Board of Thoracic Surgery - 1953
Military Service:	Lt. J.G. MC USNR, July 1945 - August 1946 Lt. MC USNR, October 1952 - July 1954 (Chief of Thoracic Surgery, U. S. Naval Hospital, St. Albans, N.Y., August 1953 - July 195
Marital Status:	REDACTED R R
Lincensure:	Illinois - 1946; New York - 1955; Colorado - 1960
Professional Societies:	

REDACTED REDACTED

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13. Publications

Giles F. Filley, M.D.

Wagner, W.W., Barker, D.B., and Filley, G.F.: A Photographic Method for Quantitating Blood Flow in the Pulmonary Microcirculation. J. Biological Photographic Assoc. 35: 95, 1967.

Filley, G.F.: Acid-Base and Blood Gas Regulation. Lea & Febiger, Philadelphia, March 1971.

Horsfield, D., Dart, G., Olson, D.E., Filley, G.F., and Cumming, G.: Models of the Human Bronchial Tree. J. Appl. Physiol. 31: 207, 1971.

Silvers, G.W., Maisel, J.C., Petty, T.L., Filley, G.F., and Mitchell, R.S.: Central Airway Resistance in Excised Emphysematous Lungs. Chest 61: 603, 1972.

Olson, D.E., Sudlow, M.F., Horsfield, K., and Filley, G.F.: Convective Patterns of Flow During Inspiration. Arch. Int. Med. 131: 51, 1973.

George W. Paul, Ph.D.

Paul, G.W., and Marc de Chazal, L.E.: Interfacial Tensions in Ternary Liquid-Liquid Systems. J. Chem. Engr. Data 12: 105, 1967.

Paul, G.W., and Marc de Chazal, L.E.: Correlation of Interfacial Free Energy in Binary and Ternary Systems. Ind. Engr. Chem. Fundamentals 8: 104, 1969.

Shoemaker, P.D., Paul, G.W., and Marc de Chazal, L.E.: Surface Tension of Simple Liquids from the Radial Distribution Function. J. Chem. Phys. 52: 491, 1970.

Paul, G.W., and Froning, H.R.: Salinity Effects in Micellar Flooding. J. Pet. Tech. 24: 957, 1973.

G. Wayne Silvers

Silvers, G.W., Maisel, J.C., Petty, T.L., Filley, G.F., and Mitchell, R.S.: Central Airway Resistance in Excised Emphysema Lungs. Chest 61: 603, 1972.

Maisel, J.C., Silvers, G.W., George, M.S., Dart, G.A., Petty, T.L., and Mitchell, R.S.: The Significance of Bronchial Atrophy. Am. J. Path. 67: 371, 1972.

Silvers, G.W., Maisel, J.C., Petty, T.L., and Mitchell, R.S.: Reduction in Peripheral Airway Resistance in Excised Emphysematous Lungs. Proc. 15th Annual Aspen Emphysema Conf. Chest 63: 32S, 1973.

Silvers, G.W., Maisel, J.C., Petty, T.L., Filley, G.F., and Mitchell, R.S.: Increase of Flow in Excised Emphysematous Lungs Following Lavage with Acetylcysteine or Salina. Am. Rev. Resp. Dis. 110: 170, 1974.

Silvers, G.W., Maisel, J.C., Petty, T.L., Filley, G.F., and Mitchell, R.S.: Flow Limitation During Forced Expiration in Excised Human Lungs. J. Appl. Physiol. 36: 737, 1974.

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Giles F. Filley, M.D. (Principal Investigator)	30%	
George W. Paul, Ph.D. Postdoctoral Res. Fellow	100%	R
G. Wayne Silvers, Research Associate	50%	
Melvin M. Newman, M.D., Consultant	Not stipulated	--
Technical		
Marlyce George, Research Technologist	50%	R
Fringe Benefits		R
	Sub-Total for A	REDACTED
B. Consumable supplies (by major categories)		
Chemicals & fixatives		750
Valves, tubing, glassware		350
X-ray room use fee & supplies		450
Recorder paper & developer		300
Suction filter		25
	Sub-Total for B	1,875
C. Other expenses (itemize)		
Publication costs		500
Maintenance and Modification of Equipment		1,000
Travel		500
	Sub-Total for C	2,000
	Running Total of A + B + C	35,573
D. Permanent equipment (itemize)		
1 ea Model 1210 Harvard Peristaltic Pump		625
1 ea Model 550 Harvard Pump speed modulator		225
1 ea Large speciman plethysmograph		200
1 ea duNouy Tensiometer		495
1 ea Cathetometer telescope and stand		370
1 ea Gionometer Eyepiece		237
1 ea Statham transducer		350
1 ea Universal illuminator		111
	Sub-Total for D	2,613
E. Indirect costs (15% of A+B+C)	E	5,336
	Total request	43,522

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	1,500	2,000	--	5,664	43,427
Year 3		1,650	1,000	--	5,955	45,656

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Source: https://www.industrydocuments.ucsf.edu/docs/mgdm0000

R: REDACTED MATERIAL

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Cardiopulmonary Physiology	USPHS 3 T01 HL05450-14S1	36,423	4/1/74 - 6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Human Pulmonary Surfactant Function in Situ	USPHS 1 R01 HL 17544-01A1	42,245	4/1/75 - 3/31/76
Surface Chemistry and Physics of Pulmonary Surfactant (Young Investigator) G.W. Paul	USPHS 1 R23 HL 18353-01	15,000	7/1/75 - 6/30/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Giles F. Filley, M.D.

Signature [signature] Date [illegible]

Telephone REDACTED
Area Code Number Extension

Checks payable to

iversity of Colorado Medical Center
Office of Grants & Contracts

Mailing address for checks

4200 East Ninth Avenue

Denver, Colorado 80220

Responsible officer of institution

Typed Name Harry P. Ward, M.D.

Title Acting Vice President for Health Affairs

Signature [signature] Date [illegible]

Telephone REDACTED
Area Code Number Extension

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

CTR Grant #

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., Inc.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Project Title: Human Pulmonary Surfactant Function In Situ

We hereby certify that human subjects involved in this proposal to whom we administer investigational or any other procedures, including personality tests and questionnaires will have signed legal consent forms, and that human subjects below the age of 18 years will have legal consent forms signed by the parents, legal guardians, or probate court. If this is deemed unnecessary or undesirable in this particular instance, we state the reasons below.

1/21/75
Date

Signature of Principal Investigator or Program Director

1/22/75
Date

Business Officer of Appropriate Institution Authority
Harry P. Ward, M.D.
Acting Vice President for Health Affairs

Return ONE copy to: The Council for Tobacco Research-U.S.A., Inc.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

#1014-GLEICH

1003546294

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

December 31, 1974

Grant Application No. 1014

PULMONARY

To: The committee comprising Drs. Feldman, Liebow and Sommers

Subject: G. J. Gleich, M.D., Mayo Clinic and Foundation, Rochester, Minnesota
New application No. 1014
"Hypersensitivity to Antigens from Tobacco as a Factor in the Pathogenesis of Chronic Bronchitis"

History

An informal inquiry was handled as Case No. 305.

Request:

Application No. 1014 requests $8,345 for one year.

Documents Submitted (attached)

1. Application dated November 19, 1974 (11 pages) including C.V.'s of Dr. Gleich and Dr. McDougall.

2. Unsigned consent form.

D.S.

DS:wg
Encl.

10035462295

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 11/19/74

1. Principal Investigator (give title and degrees):

G. J. Gleich, M.D.
Consultant in Medicine, Mayo Clinic and Mayo Foundation
Associate Professor of Internal Medicine and Microbiology, Mayo Medical School

2. Institution & address:

Mayo Foundation
200 First Street Southwest
Rochester, Minnesota 55901

3. Department(s) where research will be done or collaboration provided:

Department of Medicine and Allergic Disease Research Laboratory

4. Short title of study:

Hypersensitivity to Antigens from Tobacco As a Factor in the Pathogenesis of Chronic Bronchitis

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: 12 months

7. Brief description of specific research aims:

We will determine whether antigens are present in tobacco and tobacco smoke when these materials are used to immunize rabbits. We will also determine whether antigens present in tobacco or tobacco smoke are able to stimulate immune responses in humans with chronic bronchitis.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

8. Brief statement of working hypothesis:

Although chronic bronchitis and pulmonary emphysema are associated with chronic exposure to tobacco smoke, the mechanism by which these diseases are caused remains obscure. The effects of smoking tobacco likely include 1) a primary irritant effect on the bronchi themselves, possibly mediated by small molecular weight materials derived from the smoke, 2) the presence in tobacco smoke of materials which alter the function of host resistance mechanisms, particularly of alveolar macrophages (1), and 3) a deficiency in the flow of mucous such that the clearing mechanism of the lung is interfered with and the organ is thus prone to colonization by bacteria. These are the possibilities which have been considered most strongly in the pathogenesis of chronic bronchitis (2-6). Another possibility is that tobacco smoke may contain materials which are capable of functioning as antigens in the lung and which incite a chronic immunologically mediated inflammatory reaction. Positive immediate wheal and flare reactions to tobacco and tobacco smoke extracts (7) and the demonstration of what appears to be IgE antibodies by passive transfer (Prausnitz-Kustner method) (8,9) suggest that in some individuals there may be an immune response to constituents of tobacco or tobacco smoke (10).

9. Details of experimental design and procedures (append extra pages as necessary)

RESEARCH PLAN

A. Analysis of Extracts of Tobacco

1. The first step of the experiments will be to prepare an extract of commercial cigarette tobacco. This will be accomplished by stirring cigarette tobacco in buffered saline at room temperature for twenty-four hours. Toluene will be added for bacteriostasis. The mixture will be centrifuged and the supernatant decanted and saved. The supernatant will be analyzed for protein content by precipitation with trichloracetic acid, solubilization in dilute alkali and biuret testing. Electrophoresis of the solubilized precipitate on polyacrylamide gel at acid and alkaline pH and by isoelectrofocusing will be performed and finally the supernatant will be fractionated by gel filtration and ion exchange chromatography to isolate any major antigens.

2. A portion of the tobacco extract will also be mixed with Freund's complete adjuvant and injected into rabbits. The animals will be bled at three weeks and the serum tested for antibodies to tobacco extract by immuno-precipitation. If no antibodies are detected or if they are present only in low titer, the rabbits will be re-injected with tobacco extract plus Fruend's adjuvant and test bleedings carried out at appropriate intervals. When a satisfactory titer of precipitating antibody is present in the serum, a larger sample of serum will be collected and frozen.

3. Attempts will next be made to characterize the antigen or antigens present in tobacco extract. Using the rabbit antibody produced, the number and charge of the antigens will be determined by immunoelectrophoresis and crossed-immunoelectrophoresis (13).

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

B. Analysis of Extracts of Tobacco Smoke

The second step of our investigation will be to prepare a tobacco smoke extract. This will be accomplished by bubbling cigarette smoke through buffered saline at room temperature. The extract will be analyzed as described above for protein content and characteristics of proteins, if any, are present. The smoke extract will be mixed with Freund's complete adjuvant and injected into rabbits in an attempt to stimulate antibody formation. If present, antigens will be characterized using the methods described above for analysis of antigens in the tobacco extract.

C. Analysis of Extracts of Tobacco Smoke Exposed to Human Serum Albumin

The third step in these experiments will be to prepare an extract of cigarette smoke using buffered saline containing human serum albumin. The rationale for this experiment is the possibility that reactive chemicals in smoke may interact with groups on serum albumin, and introduce new antigenic determinants into the albumin molecule. If so the new antigenic determinants may be detected by immunization of rabbits with the smoke-extract-albumin mixture. Accordingly this material will be dialyzed and injected into rabbits. The resulting antiserums will be studied by immunodiffusion and immunoelectrophoresis to determine whether new antigenic determinants are present on the smoke-altered human serum albumin. If antigenic determinants are detected, we will determine whether there are any common antigens among the various extracts. For this study the antiserums to the smoke-extract will be absorbed with human serum albumin to render it specific for the new antigenic determinants. The absorbed serum will then be used in the experiments to determine if common antigens are present.

D. Analysis of Human Serum and Secretions

In the final phase of the experiment we will test human serum and bronchial secretions of smokers collected at the time of bronchoscopy (see attached consent form) for the presence of antibodies against tobacco extract, tobacco smoke extract and tobacco smoke-albumin extract. Immunodiffusion, complement fixation and possibly radioimmunoprecipitation procedures will be used to determine whether such antibodies are present. We recognize that the concentration of antibodies in human serum and secretions may be low. Accordingly we will use radioimmunosorption experiments in which the various smoke derived antigens are insolubilized by reaction with cyanogen-bromide activated microcrystalline cellulose (11). Following reaction of serum or secretions with the solid phase antigen and washing the resulting complex will be exposed to radiolabelled affinity-chromatography-purified antibody to IgA or IgG. This procedure, similar in principle to the radioallergosorbent test (12), is sufficiently sensitive to detect antibodies in the subnanogram (per ml) range. Therefore it should be possible to determine whether antibodies are present in the human specimen.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The laboratory facility we have at our disposal, termed the Allergic Diseases Research Laboratory, consists of 1400 sq. ft. of space we alone occupy, and another 500 sq. ft. of shared space and contains all major items of equipment for immunochemical investigations. These include cold laboratories, adequate cold storage and deep freeze space, both -20° and -70°, balances, chromatographic equipment, fluorescent microscopes, ultracentrifuges, both preparative and analytical, polarimeters, equipment for analytical electrophoresis, in all commonly used supporting media, incubators, spectrophotofluorometers and spectrophotometers. Quarters for housing experimental animals, both small animals, such as rabbits and guinea pigs, and large animals, such as goats, horses, and monkeys are available. Special laboratories (hot lab) with remote handling facilities for experiments involving radioactive isotopes, a monitoring system, and dark room facilities are available. We also have an automatic gamma counting system with two channels, an electronic calculator for background subtraction, automatic calculations of counts per minute and ratio computations, and a teletypriter print out. Finally as part of earlier studies on penicillin allergy (92) we have stocks of serum from rabbits immunized with the BPO determinant to aid in experiments with this determinant.

11. Additional facilities required:
NONE

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

R: REDACTED MATERIAL

Name: Gerald J. Gleich

Title: Consultant in Microbiology, Mayo Clinic and Mayo Foundation; Associate Professor of Internal Medicine, Immunology, and Microbiology, Mayo Medical School

Date of Birth: REDACTED

Place of Birth: REDACTED REDACTED

Present Nationality:

Sex: Male

Education: Institution	Degree	Year
Michigan College of Mining and Technology University of Michigan University of Michigan Medical School	B.A., M.D.	REDACTED

Honors: Phi Beta Kappa; Phi Kappa Phi; Alpha Omega Alpha; Sigma Xi

Relationship to Proposed Project: Principal Investigator

Research and/or Professional Experience:

Consultant in Microbiology, Mayo Clinic and Mayo Foundation	1965-Present
Associate Professor of Internal Medicine, Immunology and Microbiology, Mayo Medical School	1974-Present
Associate Professor of Internal Medicine and Microbiology, Mayo Medical School	1973-1974
Assistant Professor of Internal Medicine and Microbiology, Mayo Graduate School of Medicine	1968-1973
Instructor in Medicine, Mayo Graduate School of Medicine	1965-1968
Instructor in Medicine and Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York	1963-1964
Private Practice of Internal Medicine, Strong Memorial Hospital, Rochester, New York	1963-1964
Assistant Physician, Strong Memorial Hospital, Rochester, New York	1961-1965
Trainee in Allergy and Immunology, University of Rochester Medical Center, Rochester, New York	1961-1963
Residency, Jackson Memorial Hospital, Miami, Florida	1959-1961
Internship, Philadelphia General Hospital, Philadelphia, Pennsylvania	1956-1957

Publications

1. Griffiths, R.W., Gleich, G.J.: Proteolytic Degradation of IgG and Its Relation to Molecular Conformation. J. Biol. Chem. 247:4543-4548, July, 1972.

2. Gleich, G.J., Maldonado, J.E. and Loegering, D.A.: Identification of a Major Basic Protein in Guinea Pig Eosinophil Granules. J. Exp. Med. 137:1459, 1973.

3. Fair, D.S., Gleich, G.J., Krueger, R.G. and Kyle, R.A.: Studies on IgA and IgG Monoclonal Proteins Derived from a Single Patient. I. Evidence for Shared Individually Specific Antigenic Determinants. J. Imm. Vol. 112:201, Jan., 1974.

4. Gleich, G.J., Jones, R.T., Larson, J.B. and Baer, H.: Measurement of the Potency of Allergy Extracts by Their Inhibitory Capacities in the Radioallergosorbent Test. J. Allergy and Clin. Immunology, Vol. 53:148, March, 1974.

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5. Gleich, G.J., Loegering, D.A., Kueppers, F., Bajaj, S.P. and Mann, K.G.: Physiochemical and Biological Properties of the Major Basic Protein from Guinea Pig Eosinophil Granules. The Journal of Experimental Medicine, August 1, 1974, Vol. 140, No. 2, pp 313-332.

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

R: REDACTED MATERIAL

Name: John C. McDougall, M.D.

Title: Research Fellow, Mayo Clinic and Mayo Foundation

Date of Birth: REDACTED

Place of Birth: REDACTED

Present Nationality: REDACTED

Sex: Male

Education	Institution	Degree	Year
	University of North Dakota	B.S. (Chemistry)	
	University of North Dakota	B.S. (Medicine)	
	Bowman Gray School of Medicine	M.D.	REDACTED

Research and/or Professional Experience

Rotating Internship, Weld County General Hospital	1968-1969
Resident in Internal Medicine, Mayo Graduate School of Medicine	1971-1973
Trainee in Thoracic Disease, Mayo Graduate School of Medicine	1973-Present

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited"). Professional (give % time of investigator(s) even if no salary requested)		% time	Amount
G. J. Gleich, M.D.	Prin. Invest.	--	--
J. C. McDougall, M.D.	Co-Investigator	25%	R
Payroll Benefits (15.8%)			R
Technical			
Sub-Total for A			REDACTED
B. Consumable supplies (by major categories)			
Reagents and Glassware			1,000
Radioisotopes			200
Animals: Purchase (18) and maintenance (6 mo.) of rabbits			1,475
Sub-Total for B			2,675
C. Other expenses (itemize)			
Sub-Total for C			--
Running Total of A + B + C			6,730
D. Permanent equipment (itemize)			
Sub-Total for D			
E. Indirect costs (15% of A+B+C)		E	1,615
15. Estimated future requirements:		Total request	8,345

	Salaries	Consumable Suppli	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Functions of Eosinophils	AI 09728	144,000	5/1/70-4/30/75
Allergic Disease Center Program Grant	AI 11483	109,435	6/1/73-5/31/78
Measurement of the Potency of Allergen Extracts by The Radioallergosorbent Test	FDA 73-164	66,080	4/1/72-3/31/75
Establishment of an Allergen Certification Laboratory	NO1 AI 42546	120,430	6/25/74-6/24/76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Functions of Eosinophils (Renewal)	AI 09728	249,385	5/1/75-4/31/80

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name G. J. Gleich

Signature [signature] Date 11/27/74

Telephone 507 (Area Code) 282-2511 (Number) / 2351 (Extension)

Checks payable to

K. J. Ladner, Mayo Foundation

Mailing address for checks

200 First Street Southwest
Rochester, Minnesota

Responsible officer of institution

Typed Name K. J. Ladner

Title Treasurer

Signature [signature] Date 11/27/74

Telephone 507 (Area Code) 282-2511 (Number) / 2983 (Extension)

10035463O3

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

SIGNIFICANCE

The significance of these studies rests upon the known importance of chronic bronchitis as a cause of morbidity and mortality in the American population. We believe the studies are of theoretical interest in that they will show whether or not there are antigens present in tobacco or tobacco smoke and whether these antigens might incite an immune response in patients. Additional studies which would follow upon a positive result in the initial studies would obviously include the investigation of the effect of these antigens on the lung of experimental animals and their ability to incite inflammatory reactions in the lung as a result of their antigenicity. Were we to identify antigens in tobacco smoke, the characteristics of these materials might suggest ways to rid tobacco of the critical antigens. Alternatively if haptens were to be found we would investigate their ability to provoke inflammation in the lung and investigate ways in which these might be removed from tobacco smoke. Finally, if indeed immunologic reactions to tobacco antigens were shown, it might be possible to utilize the antibodies produced by the host as an index of the extent or severity of the inflammation occuring in the lungs, and thus as a diagnostic test of the potential severity of the pathologic process. This would provide us with a new diagnostic tool for the identification of individuals at greatest risk from inhalation of tobacco smoke.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

DESCRIPTION FOR LAY INFORMATION

A series of experiments will be performed to determine whether or not tobacco smoke when inhaled into the lungs may cause disease of the lungs by stimulating an allergic or immunologic response to the tobacco smoke. Studies of a preliminary nature will be performed using animal subjects. If these are promising, studies of human blood and bronchial secretions will also be performed to seek clues as to how the body's own defense mechanism might be turned against itself to produce chronic bronchitis and emphysema, two major health problems in America today.

1003546305

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

BIBLIOGRAPHY

1. Green, Gareth M.: Lung defense mechanisms. Med. Clin. N. A. 57:547-562, 1973.

2. United States Department of Health, Education and Welfare. The Health Consequences of Smoking: A report of the Surgeon General, 1972. DHEW Publication No. HSM 72-7516) Washington, D.C., Government Printing Office, 1972, Chapter 3.

3. Anderson, A.E., Jr., Hernandez, J.A., Holmes, W.L. et al: Pulmonary Emphysema: prevalence, severity and anatomical patterns in macrosections with respect to smoking habits. Arch. Environ. Health 12:569-577, 1966.

4. Petty, T.L., Ryan, S.F., Mitchell, R.S.: Cigarette smoking and the lungs: relation to post-mortem evidence of emphysema, chronic bronchitis and black lung pigmentation. Arch. Environ. Health 14:172-177, 1967.

5. Auerbach, O., Hammond, E.C., Garfinkel, L., et al: Relation of smoking and age to emphysema: whole lung section study. N. Eng. J. Med. 286:853-857, 1972.

6. Spain, D.M., Siegel, H., Bradess, V.A.: Emphysema in apparently healthy adults: smoking, age and sex. JAMA 224:322-325, 1973.

7. Pipes, D.M.: Allergy to tobacco smoke. Ann. of All. 3:277-282, 1945.

8. Harkavy, J., Witebsky, E.: Studies of specificity in multiple hypersensitiveness by quantitative titration and absorption of reagins. J. of All. 6:437-447, 1934-1935.

9. Peshkin, N.M., Landy, L.H.: Cutaneous reactions to tobacco antigen in allergic and nonallergic children with the direct and indirect (local passive transfer) methods of testing. The J. of All. 10:241-245, 1938-1939.

10. Silvette, H., Larson, P.S., Haag, H.B.: Immunological aspects of tobacco and smoking. Am. J. of the Med. Sci. 233:561-589, November 1957.

11. Yunginger, J. W., Gleich, G.J.: Comparison of the protein-binding capacities of cyanogen bromide-activated polysaccharides. The J. of Allergy and Clin. Immunol., Vol. 50, No. 2 109-116, 1972.

12. Yunginger, J.W., Gleich, G.J.: Seasonal Changes in IgE Antibodies and Their Relationship to IgG Antibodies during Immunotherapy for Ragweed Hay Fever. 52: 1268-1275, May, 1973.

13. Ganrot, P.O.: Crossed Immunoelectrophoresis. Scand. J. Clin. Lab. Invest. 29:39-41, 1972. Suppl 124.

1003546306

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

CONSENT

_____, the undersigned is willing to participate in a study of lung disease, under the direction of Dr. G. J. Gleich and associates, Mayo Clinic and Mayo Foundation.

I acknowledge that the following has been discussed with me in detail:

1. That as a result of my participation in this study fifteen milliliters of whole blood (about one-half ounce) will be taken from me.

2. That the removal of blood involves inserting a needle into a vein and that this may be uncomfortable.

3. That a sample of my bronchial secretions (phlegm) will be taken during the bronchoscopy procedure which I will have performed for other medical reasons.

4. That the collection of bronchial secretions involves no added risk since they are normally removed and discarded during bronchoscopy.

5. That this study has as its goal the uncovering of new information which may help in the understanding of the relationship between tobacco smoking and some forms of lung disease. This information is not likely to benefit me directly, though it conceivably might.

6. That any inquiries I have concerning this study have been answered, and I have been informed that I am free to withdraw my consent and to discontinue participation in this study, project or activity at any time, without prejudice to my care.

_____
Signed

_____
Investigator

_____
Date

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

#547E-GUARNERI

1003546308

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

January 28, 1975

Subject: Joseph J. Guarneri, Ph.D., Long Island Jewish - Hillside Medical Center
Continuation Application No. 547E
"The Influence of Cigarette Smoke on Intrapulmonary Inactivation of Inhaled Bacteria by Alveolar Macrophages"

An extensive background report including previous work done (58 pages) has been submitted to the committee but not included in the bulky agenda book.

D.S.

DS:wg

1003546309

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

January 28, 1975

Grant Application No. 547E

PULMONARY

To: The committee comprising Drs. Jacobson, Liebow, and Wyatt

Subject: Joseph J. Guarneri, Ph.D., Long Island Jewish - Hillside Medical Center
Continuation Application No. 547E
"The Influence of Cigarette Smoke on Intrapulmonary Inactivation of Inhaled Bacteria by Alveolar Macrophages"

History:

CTR support of this investigator goes back to 1960.

In March 1972 the SAB awarded a renewal grant (No. 547CR2) in the amount of $22,944 for the period July 1, 1973 through June 30, 1974, without assurance of further support.

In March 1974 the SAB denied an application for continuation of the studies on the basis that funding was not justified until a significant amount of his previously collected data was published.

An extention to March 31, 1975 without additional funds was granted.

The investigator now has three new abstracts to his credit, one full paper in press, and one paper prepared for press (references 24, 26, 27, 22 and 25, respectively - see Addendum 2); copies are enclosed.

A third full paper entitled "Influence of Acute Exposure to Cigarette Smoke of Bacterial Clearance by the Murine Respiratory Tract" is being prepared.

The enclosed request is for a continuation of the work.

Request

Application No. 547E requests $45,713 for the first year of a three year project.

1003546310

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Joseph J. Guarneri, Ph.D. January 28, 1975

Documents submitted (attached)

1. Application dated December 23, 1974 (31 pages, including C.V.'s of Drs. Guarneri and Shidlovsky).

2. Addendum 1 - an extensive background report including previous work undertaken (58 pages including Figures and Tables).

3. Addendum II (2 pages of Personal publications).

4. Two papers and three abstracts.

D.S.

DS:wg
Encl.

1003546311

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

547E

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JAN 2 3 1975

Application for Research Grant
(Use extra pages as needed)

Date: Dec. 23, 1974

1. Principal Investigator (give title and degrees):
Joseph J. Guarneri, Ph.D., Attending Microbiologist, Director Microbiology Labs., Long Island Jewish-Hillside Medical Center/Queens Hospital Center Affiliation and Associate Clinical Professor of Pathology, State University of N.Y. at Stony Brook.

2. Institution & address:

Long Island Jewish-Hillside
Medical Center
New Hyde Park, N.Y. 11040

Research Performance Site & Address
Long Island Jewish-Hillside Medical
Center/Queens Hospital Center Affiliation
82-68 164th Street, Jamaica, N.Y. 11432

3. Department(s) where research will be done or collaboration provided:

Division of Microbiology, Department of Laboratories.

4. Short title of study: The Influence of Cigarette Smoke on Intrapulmonary Inactivation of Inhaled Bacteria by Alveolar Macrophages.

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: 3 years

7. Brief description of specific research aims: The proposed project is an in vivo investigation of acute and extended exposure to whole cigarette smoke and its vapor phase on the ability of alveolar macrophages to ingest and destroy inhaled bacteria. Methods are used which permit an evaluation of:

(1) The effect of cigarette smoke on lung clearance of bacteria as related to the rate of ingestion of inhaled bacteria by pulmonary macrophages.

(2) In vitro methods for studying phagocyte-bacterium interactions will be used to determine the effect of in vivo exposure to cigarette smoke on (a) chemotactic responsiveness to a bacterial stimulus, (b) adherence of bacteria to the surfaces of macrophages, and (c) the rate at which phagocytized bacteria are destroyed.

(3) Studies will be performed to delineate the effect of cigarette smoke on the contributions of bronchial washings and of pulmonary secretions (Secretory IgA) to the antibacterial activity of alveolar macrophages.

(4) Studies are also presented to assess the influence of smoke inhalation on the metabolic activities of alveolar macrophages in phagocytosis and destruction of bacteria.

1003546312

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8. Brief statement of working hypothesis:

The current grant proposal is based on evidence which demonstrates the following: (a) alveolar macrophages play a key role in intrapulmonary inactivation of inhaled bacteria, (b) bronchopulmonary secretions (IgA) and bronchial washings contribute to pulmonary defense and may act as mediators of effective alveolar macrophage function. (c) studies aimed at providing a meaningful evaluation of the effect of tobacco smoke on pulmonary defense mechanisms are best performed under in vivo conditions of smoke exposure. The investigations proposed herein emanate from observations made in this laboratory which indicate that acute and extended (15 days) exposures to cigarette smoke cause an impairment of lung clearance of bacteria that is reversible within hours after the cessation of smoke exposure. In addition, smoke inhalation provokes a selective mobilization of alveolar macrophages without interfering with the mobilization of macrophages in response to the inhalation of bacteria. Similarly, in vivo smoke exposure results in a reversible suppression of the phagocytic ability of alveolar macrophages. Finally, the importance of macrophage mobilization in protecting the lung against cigarette smoke was also demonstrated. These findings indicate the need for further investigations and for studies of the influence of cigarette smoke as relates to the immunological and biochemical properties of alveolar macrophages in the phagocytosis of bacteria.

See pages 16 - 33 for a detailed report of the Background Material and Supporting Data that serve as the basis for the studies proposed in this grant.

9. Details of experimental design and procedures (append extra pages as necessary)

See pages 2A (1-15)

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9. Experimental Design: Study of the Influence of Cigarette Smoke on Disposal of Inhaled Bacteria by the Respiratory Tract.

Reference Response System: Clearance of *Staphylococcus aureus* by the Respiratory Tract

Sites of Respiratory Tract clearance to be studied.	Trachea and Major Bronchi (Tracheal Clearance Studies)	Lungs (Lung Clearance Studies)
Major Respiratory Tract Defense System Operative	Mucociliary apparatus	Alveolar Macrophage System
Particle Size of Bacterial Aerosol Required for Proposed Studies	5.0μ to 0.5μ	
Aerosol Generating unit to be used	DeVilbiss No. 40 Nebulizer	

Alveolar Macrophage System

Parameters of Alveolar Macrophage Activity to be studied	Mobilization	Phagocytosis
	(a) Chemotaxis (b) Migration rates (c) Cell adhesiveness	(a) Attachment of bacteria to macrophage surface (b) Ingestion of bacteria by macrophages (c) Intracellular destruction of ingested bacteria by macrophages

Areas of Comparative Assays by Laboratory

Immunologic Medicators of the Phagocytic Act	Metabolic Indices of the Phagocytic Act	Metabolic Indices of Bactericidal Activity
(a) Serum factors (b) Bronchial washings (c) Pulmonary secretions (SIgA)	(a) Respiration (b) Glucose metabolism (c) ATPase activity	(a) Catalase and peroxidase activity and H_2O_2 production (b) Hydrolytic enzyme activity

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Experimental Procedures:

All studies will be performed under in vivo conditions of smoke-exposure (See Methods of Procedure Page 2A-8)

A. Observations of Tracheal and Lung Clearance: The following specific information is sought from these studies: (a) the effect of extended exposure to whole cigarette smoke and its gas-phase on tracheal and lung clearance of bacterial deposits and (b) the reversibility of any adverse change in clearance rates attributable to smoke-exposure. For this purpose, experimental and control mice will be first exposed to cigarette smoke and a secondary air flow, respectively, and then challenged with aerosols of S. aureus for 30 minutes. Immediately following aerosol exposure (0 time) some of the smoke-exposed and control animals will be sacrificed to determine the number of bacteria initially deposited in their trachea and lungs. The remainder will be killed 15 min, 30 min, 1 hr, 2 hrs and 4 hrs after bacterial challenge to ascertain the number of viable bacteria residing in the trachea and lungs of smoke-exposed and control mice. From these data, it is possible to calculate the rate at which bacteria are cleared from the trachea and lungs. These studies will be performed in mice exposed daily for 1.0 hour to cigarette smoke over a 3 week period. Tracheal and lung clearance rates will be measured immediately following and 24 to 48 hrs after exposure to cigarette smoke for 1, 5 and 15 days. (See Methods of Procedure Pgs. 2A(8-9)-).

B. Alveolar Macrophage Activity: These studies will be performed under the same conditions of smoke-exposure used to assess the effect of extended exposure to whole and gas-phase cigarette smoke on tracheal and lung clearance. In this regard, mice and/or rabbits will be exposed daily for 1.0 hr to cigarette smoke over a 3 week period and alveolar macrophage activity will be assessed immediately following and 24-48 hrs after daily exposures to smoke for 1, 5 and 15 days, respectively.

(1) In Vivo Phagocytosis and Intracellular Killing Activity: Studies will be performed to determine the effect of extended exposure to whole and gas-phase cigarette smoke on the antibacterial activity of alveolar macrophages in the intact and functioning lung. For this purpose experimental and control mice will be first exposed to cigarette smoke and a secondary air flow, respectively, and then challenged with staphylococcal aerosols for 30 minutes. Immediately after bacterial challenge (0 time) and at hourly intervals during a 4 hour post-aerosol exposure period, alveolar macrophage harvests obtained from smoke-exposed and control animals will be processed as follows to obtain data on phagocytosis and intracellular killing: bacterial counts will be obtained from an aliquot of the total lung washout and the remainder will be separated by differential centrifugation at 1500 rpm for 15 min into a supernatant fraction containing free bacteria and a cellular fraction laden with alveolar macrophages and phagocytized bacteria. The number of viable bacteria present in each fraction will be determined by a standard pour plate technique. The decrease in the number of viable bacteria present in the total lung washout, as a function of time will be used as an index of the clearance rate of the lung sample obtained by the lavage technique. Similarly, a decrease in the viable counts of the supernatant and cellular fractions as a function of time, will be used as a measure of the ingestion and intracellular killing of bacteria by alveolar macrophages. (See Lung Harvest Method p. 2A-9).

In a second series of studies groups of smoke-exposed and control mice will be exposed to staphylococcal aerosols as outlined above. At 0 time, 1, 2 and 4 hrs after infection, groups of control and smoke-exposed animals will be sacrificed and the lungs of each mouse exposed and perfused with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 via polyvinyl chloride catheter threaded into the distal tracheal segment. The fixed lung will be embedded in paraffin and sections

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cut at 4 to 5 μ. The sections will be stained with Brown and Brenn tissue stain for bacteria (Preece, A. Manual for histologic techniques. 1972. Little, Brown & Co. Boston, p 320) and scanned for staphylococci with a Leitz Orthoplan microscope. The intra and extracellular location of 500 consecutive bacteria will be determined and used as a measurement of the proportion of inhaled bacteria phagocytized by control and smoke-exposed mice (Goldstein et al, J.Clin. Invest., 54:519, 1974). These studies will be performed under the same conditions of smoke-exposure as used to assess the effect of extended exposure to whole and filtered cigarette smoke on bacterial clearance. In this manner, antibacterial activity will be assessed immediately following and 24 hours after exposure to cigarette smoke for 1, 5 and 15 days. Under these conditions it will be possible to compare the effect of whole and gas-phase cigarette smoke on the rate of ingestion and destruction of inhaled bacteria by alveolar macrophages and to assess the relative influence of the particulate and gas phases of smoke on these important parameters of macrophage function.

(2) In Vitro Phagocytosis and Intracellular Killing Activity: In a separate series of studies, to determine the percent bacteria phagocytized, percent intracellular survival of phagocytized bacteria and percent bacteria killed by macrophages, alveolar macrophages harvested from smoke-exposed and control mice and rabbits will be challenged with S. aureus in an in vitro phagocytosis system. Fixed numbers of staphylococci will be added to known numbers of macrophages adhering to the flat surface of a tissue culture flask containing Hanks' solution (0.1% glucose). Immediately after bacterial challenge and at various intervals thereafter, the extracellular fraction containing free or unphagocytized bacteria will be isolated by removing the supernatant fluid from macrophage cultures with a pipette. To recover the macrophage fraction, the tissue culture flask will be vigorously shaken after adding glass beads and distilled water. Bacterial counts will be obtained from each fraction by a standard pour plate technique. The in vitro phagocytosis system and method of processing macrophage cultures used to obtain this information is presented in detail on page 23 (Addendum I).

Since phagocytosis has been shown to be a two stage event consisting of the adherence to and subsequent destruction of ingested bacteria by phagocytes, methods will also be used to differentiate bacterial attachment from ingestion. After challenging macrophages cultures with S. aureus for 1.5 hrs, the macrophage fraction will be separated from the extracellular fraction as previously described and incubated for 15 min in 3.0 ml of Hanks' solution containing 2.5% trypsin (Grand Island Biological Company). The bacteria released from the surface of macrophages by trypsin (adherence fraction) will be recovered by decanting the supernatant and washing the macrophages three times with 2.5 ml of Hanks' solution. The bacteria associated with the trypsin-treated macrophages are present within macrophages and may be referred to as the intracellular fraction of the total bacteria recovered. To recover the intracellular fraction of bacteria, the trypsin-treated macrophages will be lysed by treatment with cold sterile distilled water. The number of viable bacteria present in the adherence and intracellular fractions is determined from bacterial counts obtained from nutrient agar pour plates incubated at 37°C for 48 hrs. Under these conditions, it is possible to determine the % viable bacteria attached to (% adherence) and within alveolar macrophages (% intracellular) at various times during the phagocytic event:

$$\% \text{ adherence} = \frac{\text{No. viable bacteria in adherence fraction}}{\text{No. viable bacteria in the adherence and intracellular fractions}} \times 100$$

$$\% \text{ intracellular} = \frac{\text{No. viable bacteria in the intracellular fraction}}{\text{No. viable bacteria in the adherence and intracellular fractions}} \times 100$$

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In other studies, intracellular destruction of S. aureus by alveolar macrophages will be studied in the absence of all other aspects of the phagocytic event. For this purpose lysostaphin (Schwartz Mann) will be used (final concentration of 150 µg/ml) to kill all extracellular bacteria as well as those adhering to macrophage membranes. Lysostaphin is a muralytic enzyme which does not enter phagocytes and selectively eliminates extracellular staphylococci (Tan et al, J. Lab.Clin.Med., 78:316, 1971). Macrophage cultures are challenged with S. aureus for 1 hr at 37°C. At this time, 0.1 ml of lysostaphin is added to all macrophage cultures. After 30 minutes incubation at 37°C, duplicate flasks will be removed from the incubator and treated with 0.3 ml of trypsin to inactivate and neutralize the lysostaphin. Viable intracellular bacteria will be released by agitation with glass beads and osmolysis with cold sterile distilled water. Two hours later, duplicate flasks will be taken out of the incubator as outlined above. Viable bacterial counts will be made 48 hrs later and the % intracellular killing of S. aureus is determined as follows:

$$\% \text{ intracellular killing} = 100\% - \left[\frac{\text{No. viable bacteria in macrophage cultures 2.0 hrs after bacterial challenge period}}{\text{No. viable bacteria in macrophage cultures immediately after bacterial challenge period}} \times 100 \right]$$

(3) Influence of Other Factors: Studies are also planned to evaluate the influence of increased macrophage numbers, opsonization of bacteria, pre-treatment of bacteria with alveolar lining material, pre-treatment of bacteria with the total acellular fraction of lung harvests on the phagocytic and bactericidal powers of alveolar macrophages. In separate studies alveolar macrophages will be challenged with S. aureus and incubated at 37°C in tissue culture flasks containing Hanks' solution supplemented with the following: (a) normal serum with and without specific immune serum added, (b) alveolar lining material (ALM) alone, ALM plus normal and immune serum, ALM plus immune serum and ALM plus normal serum and (c) concentrated acellular fraction (CAF) alone, CAF plus normal and immune serum, CAF plus immune serum and CAF plus normal serum. This information is needed for proper analysis of the events observed, under in vivo conditions following an airborne bacterial challenge and is in keeping with recent observations suggesting that local immune systems and pulmonary secretions play a significant role in pulmonary defense (1,2) and, as such, may act as mediators of alveolar macrophage function. In addition, the above information will be used to establish in vitro culture conditions necessary for meaningful studies of macrophage mobilization and the metabolic activity of alveolar macrophages as outlined below; namely, in vitro culture conditions that best reflect the in vivo circumstances in the live and intact lung. (See Methods E., pgs. 2A9-10).

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(4) In Vitro Studies of Cell Adhesiveness and the Motility and Migratory Response of Basal and Mobilized Alveolar Macrophages to Chemotactic Substances: An understanding of this aspect of alveolar macrophage activity is desirable because motility and changes in cell adherence may be critical in the mobilization and migration of lung phagocytes to pulmonary sites, as well as their subsequent antibacterial activity during the normal situation and in response to the inhalation of viable bacteria and cigarette smoke. The data obtained from these studies will be correlated with the results of studies completed in this laboratory concerning the mobilization of alveolar macrophages in response to the inhalation of viable bacteria in the normal situation and during the inhalation of cigarette smoke.

For this purpose, a method of studying leukocyte motility

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reported by Carruthers (3) will be adapted to alveolar macrophages. This method is based on the ability of motile cells to move through the pores of a membrane filter. Two O-ring joints separated by a millipore filter are clamped together and sealed to form 2 distinct chambers. Fixed numbers of alveolar macrophages ($1\text{-}3 \times 10^7$) suspended in Hanks' solution (3 ml.) containing glucose will be introduced into chamber number one which is then sealed with a paraffin plug. The second chamber will be filled with 5 mg. of insoluble potato starch and plugged. After an initial period of incubation at 37°C to permit monolayer formation, the chambers will be inverted so that the test cells will then be on the bottom side of the filter, and the chemotactic substance, if present, is on the top side of the filter. The chamber will then be placed in an incubator at 37°C for 4 hours. At the end of various hourly intervals, the filter will be removed, stained with hematoxylin and subjected to microscopic study. In this way, the number of cells on both sides of the filter will be enumerated and used as an index of the migratory response elicited by stimulatory agents. Separate studies are planned to evaluate the changes in cell adhesiveness that occur in phagocytizing alveolar macrophages. The method of studying the adhesive properties of blood leukocytes reported by Allison and Lancaster (4) will be adapted to alveolar macrophages. Test tube cultures of fixed numbers of lung phagocytes suspended in Hanks' solution will be challenged with known numbers of bacteria. Under conditions favoring maximum phagocytosis, microscopic methods will be used to determine the formation of cell aggregates by phagocytizing macrophages. Changes in cell adhesiveness will be evaluated under the same experimental conditions described above to assess motility.

(5) Metabolic Studies: The proposed project will include studies of the energy metabolism, hydrolytic enzyme activity and peroxidative metabolism of basal and mobilized alveolar macrophages harvested from control and smoke-exposed animals. This information is desirable, since energy output and cell metabolism represent important potential links in the successful mobilization and subsequent antibacterial activity of alveolar macrophages. Indeed, alterations in hydrolytic enzyme activity and peroxidative metabolism may interfere with the primary immunological function of macrophages in pulmonary defense, namely, the destruction of inhaled microorganisms. The metabolic studies will be performed under the same culture conditions (suspending medium) used to assess the influence of cigarette smoke on the phagocytic and bactericidal capacities of alveolar macrophages.

Respiration studies: Experiments are planned which will permit determinations of the oxygen consumption and lactic acid content of alveolar macrophages incubated in basal medium and media supplemented with glucose plus serum with and without the presence of particles that induce phagocytosis. In separate studies serum will be supplemented with and/or replaced by alveolar lining material and the concentrated acellular fraction of lung harvests as outlined in the phagocytosis studies detailed on pgs.2A9-10. These studies will be performed with the following categories of macrophages: (a) basal macrophages harvested from control and smoke-exposed animals and (b) mobilized macrophages harvested from control and smoke-exposed animals immediately after exposure to aerosols of a phosphate buffer for 30 minutes. (see Methods of Procedure p.2A-10).

Glucose metabolism studies: Experiments with specifically labelled glucose as substrate will be performed to evaluate the effect of cigarette smoke on the metabolism of glucose by alveolar macrophages. These studies will be done with the same categories of basal and mobilized macrophages used in the respiration studies (see Methods of Procedure p. 2A-10).

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ATPase activity: Experiments are planned to compare the ATPase activity of basal and mobilized alveolar macrophages harvested from control and smoke-exposed animals. ATPase activity will be determined from the liberation of inorganic phosphate (Pi) upon incubation of alveolar macrophages with adenosine triphosphate (ATP). (See Methods of Procedure p. 2A-10)

Catalase activity, peroxidase activity and hydrogen peroxide production: Recent studies (5) have presented evidence for the presence of a catalase-dependent peroxidative metabolism. Peroxidative metabolism represents a biochemical pathway capable of increasing glucose metabolism and hydrogen peroxide formation. In this regard, published reports clearly demonstrate that phagocytosis by normal alveolar macrophages is accompanied by increased glucose metabolism (6,7) and intracellular recovery of hydrogen peroxide (5). The potential role of catalase controlled concentrations of hydrogen peroxide as an intracellular bactericidal agent has been recognized. For these reasons studies are proposed to compare the catalase activity, peroxidase activity and hydrogen peroxide production in macrophages harvested from control and smoke-exposed rabbits. These studies will be performed with basal macrophages, and macrophages mobilized in response to the inhalation of heat-killed staphylococci harvested from both control and smoke-exposed animals. (See Methods of Procedure p. 2A-11)

Hydrolytic enzyme studies: The proposed project will include a study of the enzymatic activity and intracellular distribution of a specific group of hydrolytic enzymes in basal and mobilized macrophages harvested from control and smoke-exposed animals. The enzymes acid phosphatase, lysozyme, lipase, beta-glucuronidase and cathepsin are of immediate interest because their activity is increased in the BCG induced alveolar macrophage (8,9). To accomplish these aims, the enzymatic activity detectable in the cell-free supernatant and alveolar macrophage fractions of lung harvests will be assayed. Interest in both fractions of lung harvests resides in the fact that bronchial mucus contains several poorly defined substances (10) including lysozyme (11) that exert nonspecific bacteriostatic and bactericidal activity against gram positive and gram negative bacteria as well as the potential phagocytosis promoting factor present in pulmonary secretions (1,2). In addition, alveolar macrophages by virtue of their high hydrolytic enzyme content, presence in large numbers and rapid turnover rate may contribute to the enzymatic activity found in mucus secretions.

A comparison will be made of the enzymatic activity and intracellular distribution of the above hydrolases in the following categories of macrophages: (a) basal macrophages harvested from control and smoke-exposed animals and (b) mobilized macrophages harvested from control and smoke-exposed animals immediately after exposure to aerosols of viable or heat-killed staphylococci. For this purpose, smoke-exposed and control animals will be exposed to the bacterial aerosols for 30 minutes. Enzyme determinations will be made with the lung harvests obtained from both groups of animals immediately after bacterial challenge and at hourly intervals over a 4 hour post-aerosol exposure period. The choice of laboratory animals (mice or rabbits) to be used in all enzyme studies will be governed by the minimal yields necessary for adequate enzyme assay. (See Methods of Procedure pgs. 2A-11-12)

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Experimental Methods:

A. Smoke-Exposure System: A smoke generating apparatus will be used to deliver puffed cigarette smoke under controlled conditions. In the studies proposed herein, the apparatus will be adjusted to deliver 35 ml puffs each of 2 seconds duration from non-filtered cigarettes. The instrument is equipped with a rotating disc which can accommodate 30 cigarettes at one time, so that it is possible to maintain a continuous stream of puffed smoke generated at a rate of 1 L/min. The initial smoke delivered by the cigarettes is diluted approximately 1 to 20 and transported across an animal exposure chamber by a secondary air flow. Control animals are placed in a plexiglass chamber and exposed to a secondary air flow. These are the same conditions of smoke exposure that were used to assess the influence of acute and extended exposure to puffed cigarette smoke on bacterial clearance and alveolar macrophage function in studies reported under Addendum I, Supporting Data, pages 21 to 30. In studies with filtered cigarette smoke, the smoke generating apparatus will be modified to include the insertion of a glass fiber filter disc. The tar content in the smoke will be determined by established methods. Air samples from the smoke exposure chamber will be collected on filter paper, weighed, extracted in alcohol and subjected to spectrophotometric assay.

B. Conditions of Animal Smoke-Exposure: Animals will be exposed daily for 1.0 hr for 3 weeks to whole cigarette smoke or cigarette smoke passed through a glass fiber disc. The maximum number of total days of smoke-exposure planned over the entire 3 week study period will be 15 days. However, total days or daily length of smoke-exposure will be reduced if toxicity, animal death or bacterial contamination of broncho-pulmonary tissue are noted. The daily length of exposure corresponds to the smoke-exposure periods previously used in this laboratory to study the effect of acute and extended exposure to cigarette smoke on tracheal clearance, lung clearance and alveolar macrophage activity. Under these conditions, it will be possible to determine the effect of whole and gas-vapor phase cigarette smoke on pulmonary defense and to correlate these observations with the data obtained to date under conditions of acute and extended exposure to cigarette smoke (See Addendum I, Supporting Data, pages 21 and 22.). The proposed protocol is similar to that used by LaBelle et al (12) to study the effects of acute and extended exposure to cigarette smoke on pulmonary clearance of radioactive test particles.

C. Bacterial Aerosol Exposure Unit: Since bacterial clearance studies comprise a major part of the proposed research project, a review of the method of aerosol formation, exposure and quantitative aspects of bacterial clearance is indicated. Aerosols are generated from a buffered suspension (pH 7.3) of staphylococci contained in glass nebulizers. The initial spray from the nebulizers is directed into mixing chambers through which a secondary air flow of 100 cubic feet/min is drawn. The large volume of secondary air serves to mix, dilute and dry the initial bacterial aerosol; direct it past an interposed baffle for removal of large droplets; and then carry it through a large plexiglass exposure chamber that can accommodate up to 200 mice. The particle size distribution of the bacterial aerosol is monitored with an Andersen Sampler (13). White male Swiss Webster mice are divided into groups of 5 to 10 animals and exposed to staphylococcal aerosols for 30 min. Immediately after exposure (0 time) one group of animals is sacrificed to determine the numbers of viable bacteria deposited in the trachea and lungs, respectively. The trachea and lungs are removed as separate blocks and individually ground in glass homogenizers. The remaining groups of challenged mice are killed and processed 15 min, 30 min, 1 hr, 2 hrs and 4 hrs after aerosol exposure. Bacterial counts are obtained from nutrient agar pour plates of lung and tracheal tissue homogenates. By this method paired studies of lung and tracheal clearance of bacterial deposits are possible. By subtracting the mean number of culturable bacteria retained in

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the lung at each interval during the post aerosol exposure period from the number originally deposited (0 time), the mean number of staphylococci killed by the lung may be derived. From these data lung clearance rates are calculated by expressing the number retained in the lung as a percentage of the deposition number (0 time) and subtracting this value from 100%. Similarly, from the numbers of viable bacteria present in the trachea at 0 time and at the same intervals after aerosol exposure, the numbers and percent bacteria cleared by the trachea may be obtained. It must be emphasized that the term clearance refers solely to the decline in the number of culturable bacteria remaining in the trachea and lungs after aerosol exposure.

D. Harvesting of Alveolar Macrophages: In order to correlate macrophage activity with bacterial clearance, a method has been developed in this laboratory for harvesting alveolar macrophages from the murine lung (14,15). Mice are sacrificed and the trachea and lungs exposed. The intact lung is washed by 5 successive 1.0 ml washes with Hanks' solution and the harvested cells recovered by centrifugation. By this method, it is possible to obtain 1.0 to 2.0 x 10^5 macrophages from each mouse lung with 90% viability. Total cell counts are performed in a bright line hemocytometer and differential counts are made on Wright stained smears. Cell viability is assessed by the capacity of alveolar macrophages to reject the stain Eosin Y. The number of alveolar macrophages that are available in harvest by this technique under basal conditions is referred to as the basal yield. Therefore, mobilization is taken to represent the increase in macrophage numbers over basal levels harvestable from the lungs after a bacterial challenge. The mobilization of alveolar macrophages may be quantitated by this technique. Studies completed in our laboratory to date indicate that macrophage yields are increased 1.5 times basal levels after exposure to aerosols of a phosphate buffer or dead Staphylococcus aureus (14), and 2 to 3 times basal levels in response to aerosols of viable S. aureus (14,15). During the post-aerosol exposure period there is an initial drop in macrophage numbers but elevated levels are restored in 30 min. and this increase is maintained for 4 hours (15). (See Addendum II, publication in press entitled "Clearance of Inhaled Bacteria from the Murine Respiratory Tract")

Alveolar macrophages are harvested from the lungs of albino rabbits weighing 1.0 to 2.0 kilograms by the general method of Myrvik et al (16). The animals are killed by injecting air in the marginal ear vein. This method of sacrificing animals is used to avoid any depressant effects that anesthetics may have on alveolar macrophage function. The trachea and lungs are exposed, and macrophages harvested by washing out the intact lungs with 17 ml. of Hanks' solution. The harvested cellular contents of the lungs are recovered by centrifugation at 2000 rpm for 20 minutes. Total cell counts are performed in a bright line hemocytometer, and differential counts are made on Wright stain smears. By this method, 95% of the macrophages harvested are viable as determined by the Eosin Y technique(17).

E. Acellular Fractions of Lung Harvests: Recent studies suggest that secreted fluids present in the bronchopulmonary tree may independently or in concert with alveolar macrophages play a significant role in pulmonary defense against inhaled bacteria. Alveolar lining material (ALM) has been reported to enhance the bactericidal activity of rat alveolar macrophages (1). Secretory IgA is present in the tracheobronchial washing of normal patients (2) and has the capacity to kill Escherichia coli (18) and inhibit the adherence of certain strains of streptococci to epithelial tissue (19). For these reasons, the acellular fractions of lung harvests (ACF) will be collected, concentrated, Secretory IgA levels monitored and ALM isolated. In addition, the antibacterial and phagocytic properties of concentrated acellular fraction and ALM against S. aureus will be assessed in the phagocytosis system detailed on page 23. These studies will be performed with acellular fractions of lung harvests obtained from control and smoke-exposed animals.

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The method of LaForce et al (1) will be used to isolate alveolar lining material from the lungs of rabbits. Rabbits are sacrificed by injecting air into the marginal ear vein, and the trachea and lungs are exposed. The trachea is canulated with a sterile polyethylene tube, and 17 ml of sterile heparinized saline (10 units per ml) are introduced in the lung and recovered by aspiration. The recovered bronchoalveolar saline lavage fluid is centrifuged at 800 g for 8 minutes; the supernatant decanted and saved. The cell free supernatant is centrifuged at 40,000 g at 4°C for 20 minutes. The recovered precipitated pellet represents the alveolar lining material or surfactant fraction (20).

In order to study the antibacterial and phagocytosis promoting characteristics of the entire acellular fraction of lung harvests, bronchoalveolar saline lavage fluid recovered from rabbit lungs will be initially concentrated by ultrafiltration (Diaflow Membranes, Amicon Corp) and fractionated by gel column chromatography.

The presence of Secretory IgA (SIgA) in bronchoalveolar washings will be monitored by double immunodiffusion (21) against anti-SIgA and anti-secretory piece sera. SIgA levels will be quantitated by single racial immunodiffusion (22) utilizing anti-SIgA serum impregnated in the agar gel and SIgA as the antigen standard. The SIgA antigen standard will be prepared from clarified colostrum (23). The latter procedure includes: (a) separation by gel chromatography and further purification by anion-exchange chromatography using a stepwise elution gradient of phosphate buffers of varying ionic strengths. The purity of SIgA will be assessed by disc electrophoresis (24).

F. Metabolic Studies: Areas of comparative assays to be performed.

Respiration: Measurements of oxygen uptake will be determined in a Gilson respirometer using flasks with a 15 ml capacity containing monolayers of alveolar macrophages in a total liquid volume of 3.2 ml (25). The CO_2 will be absorbed by 0.2 ml of 20% KOH in the center well. In accordance with the protocol of each study, glucose (5.0 to 10 mM) and polystrene spheres at a concentration of 2.0 to 2.5 mg/ml will be introduced via the side arm. After completion of the oxygen consumption measurements, the cells will be harvested and their lactic acid content determined by the method of Barker et al(26). For this purpose, the recovered macrophages will be washed in saline and cell extracts are to be prepared as described by Myrvik et al (27).

Glucose metabolism: The radioactive measurements will be done as described by Myrvik et al (27). Alveolar macrophages are harvested and placed in Erlenmeyer flasks containing Medium 199 without serum or glucose. In separate experiments BCG and heat killed staphylococci will be added to all flasks except the control flasks. After incubation at 37°C in a shaker bath for 1, 2, 4, 6 and 18 hours, glucose 1-C^{14} or glucose 6-C^{14} is added to the flasks, and reincubated for 1 hour. The reaction is stopped with sulfuric acid and counts obtained with a liquid scintilation counter.

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ATPase: ATPase activities will be carried out by the method outlined by Wahler et al (28). The assay medium will contain 100 mM sucrose, 30 mM glyclglycine, 30 mM imidazole, 5 mM $MgCl_2$, 2 mM ATP, protein equivalent of cells 50 to 100 ug and, as indicated, 50 mM NaCl plus 5 mM KCL (pH 7.5). In studies performed in the absence of Na^+ and K^+, the medium will contain 200 mM sucrose. Reactions will be carried out in a 2nd volume for 20 minutes at 30°C and activity will be expressed as micromoles of Pi liberated per milligram of protein per hour.

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Catalase/Peroxidase Activites and H_2O_2 production: Catalase activity will be determined by the method of Feinstein (29) using 0.1M sodium perborate as substrate. Perborate utilization in 5 min is measured by titration with a 0.1N solution of potassium permanganate after the reaction is stopped with 1N sulfuric acid. Catalase activity will be determined after incubation in Krebs Ringer Phosphate Solution containing 15% homologous serum and 5.5mM glucose at pH 7.4. Measurements will be performed on macrophage preparation after disruption by either homogenization or repeated freeze-thawing using acetone-dry ice. Both total extract and supernatant obtained by centrifugation at 8000 rpm for 10 min will be assayed. Activity will be expressed as milliequivalents of perborate utilized in 5 min, 1 U representing the utilization of 1 mEq of perborate. Peroxidase activity will be assessed by a modification of the guaiacol method of Chance and Maehly (30). Whole extracts of freeze-thawed cells will be employed. The assay medium contains 0.1 M phosphate buffer at pH 7.4, 0.5 ml of 100 mM guaiacol, 0.2 ml of extract sample, and 0.02 ml of ice-cold 10 mM H_2O_2. Absorbancy changes due to tetraguaiacol formation will be measured at 750 nm in a spectrophotometer and the time required to produce an 0.05 U increase in absorbancy recorded. Results will be expressed in reciprocal seconds per 10^9 cells.

Hydrogen peroxide will be determined spectrophotometrically on dialysates of AM as described by Paul and Shara (31). The nonfluorescent dye, diacetyl-2,7-dichlorofluorescin (LDADCF), was synthesized by the method of Brandt and Keston (32) and the fluorescence of the oxidized product of alkali-activated LDADCF was measured with an Aminco-Bowman spectrofluorimeter. The excitation wave length was 340 nm and the emission wave length 525 nm.

Hydrolytic Enzymes: The lung washings obtained from animals by the lavage technique will be separated into a cell-free supernatant fraction and cellular fraction containing alveolar macrophages by centrifugation at 4°C for 15 min at 10,000 rpm (11). The supernatant will be frozen at -60°C and stored until assay. The harvested alveolar macrophages will be washed twice in phosphate buffered saline (pH 7.2); quantitated by hemocytometer count; and viability assessed. Saline extracts of the washed cells are to be prepared for enzyme activity studies as outlined by Myrvik et al (27) cell disruption by alternate freezing and thawing for 5 consecutive cycles and removal of cellular debris by centrifugation at 2,500 rpm for 10 min at 4°C. The completeness of cell disruption will be audited by phase optics. In order to evaluate the contribution made by alveolar macrophages to the enzymatic activity detectable in the supernatant fraction, in vitro studies of the rate of release of the enzymes in question by pulmonary macrophages are planned. As outlined by Holzman et al (11) large numbers of alveolar macrophages will be suspended in tissue culture medium and samples of the cell population assayed for specific enzymatic activity after incubation at 37°C for various periods of time. In studies of the intracellular distribution of the hydrolases, alveolar macrophages will be suspended in a 0.25M sucrose solution, ruptured by homogenization and sedimented by centrifugation into four fractions (33). The nuclear fraction (N) will be sedimented by centrifugation at 250 X g; the heavy granule fraction (HG) at 5000 X g for 15 min; and the light granular fraction (LG) and supernatant fraction (S) by centrifugation at 25,000 X G. Each fraction will be submitted to 5 cycles of freezing and thawing and clarified by centrifugation at 2500 X G for 20 min and subjected to enzyme assay.

For the purpose of enzyme analysis, lysozymes will be quantitated by using suspensions of Micrococcus leisodykticus as substrate. Tests will be standardized with known amounts of crystalline egg white lysozyme and results expressed as egg white lysozyme equivalents. Acid phosphatase will be measured by the procedure of Hofstec(34) using O-carboxyphenyl phosphate as substrate. An increase in absorbance of 0.001 optical density units/min under standard conditions

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will be taken to represent a unit of activity. Beta-glucuronidase will be assayed by the procedure of Fishman et al (35) using phenolphthalein mono-betaglucuronide as substrate with reactions carried out in 0.1M acetate buffer at pH 4.5 and 38°C. One unit of glucuronidase is the activity resulting in the liberation of 1 ug/hr phenolphtalein. Lipase activity will be determined by the method of Cohn et al (8) in which naphthol laurate serves as substrate and the increase in activity caused by sodium taurocholate is taken to represent minimal lipase activity. The results are to be expressed as micromoles of naphthol liberated per hour. Cathepsin will be assayed employing a 2% solution of denatured hemoglobin as a substrate, as described by Anson (36) and protein digestion estimated with a spectrophotometer by absorption at 280 nm (33). A unit of activity being defined as the increase in optical density produced by 0.001 meq of tyrosine.

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References to Experimental Procedures And Methods

1. LaForce, F., Kelly, W. and Huber, G. : Inactivation of staphylococci by alveolar macrophages with preliminary observation on the importance of alveolar lining material. Am. Rev. Resp. Disease. 108:784, 1973.

2. Kelmowitz, R. : Immunoglobulins in normal tracheo-bronchial washings. J. Lab. Clin. Med. 63:54, 1964.

3. Carruters, B.M.I.: Method of studying normal variation effect of physical alterations in environment, and effect of iodacetate. Canad. J. Physiol. and Pharm. 44:457, 1966.

4. Allison, Jr., F. and M.G. Lancaster: Studies on the factors which influence the adhesiveness of Leukocytes in vitro. Ann. of N.Y. Acad. Science 115:936, 1964.

5. Gee, J. B. Vassallo, C.L., Bell, P., et al.: Catalase-dependent peroxidative metabolism in the alveolar macrophage during phagocytosis. J. Clinical Invest. 49: 1280, 1970.

6. Karnosky, M. L. and Sbarra, A.J. : Metabolic changes accompanying the ingestion of particulate matter by cells. Amer. J. Clinical Nutrition, 8: 147, 1960.

7. Ouchi, E. R., Selvaraj, R. and Sbarra, A.: The biochemical activity of rabbit alveolar macrophages during phagocytosis. Exp. Cell Res. 40: 456, 1965.

8. Cohn, Z.A. and Wiener, E.: The particulate hydrolases of macrophages. I. Comparative enzymology, isolation and properties, J. Exp. Med. 118:991, 1963.

9. Cohn, Z. A. and Wiener, E.: The particulate hydrolases of macrophages. II. Biochemical and morphological responses to particle ingestion. J. Exp. Med. 118: 1009, 1963.

10. Miller, T. E. and Watson, D.W.: Innate immunity: Med. Clin. N. Amer. 49: 1489, 1968.

11. Holzman, R.S., Gardner, D. E. and Coffin, D.R.: In vivo interactivation of lysozyme by ozone: J. Bacteriol. 96: 1552, 1968.

12. LaBelle, C.W., Bevilaqua, D.M. and Breiger, H.: The influence of cigarette smoke on lung clearance: Arch. Environ. Health 40: 588, 1966.

13. Andersen, A.A.: New sampler for the collection, sizing and enumeration of viable airborne particles. J. Bacteriol. 76:471, 1958.

14. Guarneri, J. J. and Laurenzi, G.A.: Mobilization of alveolar macrophages as a pulmonary defense mechanism against inhaled bacteria.: Bacteriol Proc., p. 100, 1968.

15. Guarneri, J. J. and Laurenzi, G.A.: Effect of alcohol on the mobilization of alveolar macrophages: J. Lab. Clin. Med. 72: 40, 1968.

16. Myrvik, Q. N., Leake, E.S., Fariss, B.: Studies on pulmonary alveolar macrophages from the normal rabbit: A technique to procure them in a high state of purity. J. Immunol. 86: 128, 1961.

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References to Experimental Procedures And Methods

17. Dannenberg, A. M., Burstone, M. S., Walter, P. C., and Kinsley, J.W.: A histochemical study of phagocytic and enzymatic functions of rabbit mononuclear and polymorphonuclear exudate cells and alveolar macrophages. I. Survey and quantitation of enzymes, and states of cellular activation.: J. Cell. Biol. 17:465, 1963.

18. Burdon, D.W.: Bactericidal action of immunoglobulin A. Proceeding Path. Soc. Great Britain and Ireland. 5:VII, 1972.

19. Fukui, Y., Fukui, K. and Morigama, T.: Inhibition of enzymes by human salivary immunoglobulin A. Infection and Immunity. 8:335, 1973.

20. Morgan, T.: Pulmonary Surfactant. N. Eng. J. Med. 284:1185, 1971.

21. Oucherlony, O.: Handbook of Immunodiffusion and Immochlectrophoresis. Ann Arbor Science Publications. Ann Arbor, Michigan, 1968.

22. Fahey, J. L. and E. M. McKelvey: Quantitation of serum immunoglobulins in antibody - agar plates. J. Immunol. 8:335, 1965.

23. Cebra, J. and Robbins, J.: Gamma A immunoglobulin from rabbit colostrum. J. Immunol. 92: 12, 1966.

24. Gordon, A. H.: Electrophoresis of Protein in Polyacrylamide and Starch Gel. Amer. Elsevier Publishing Co., N.Y., 1970.

25. Oren, R., Farnham, A.E., Saito, K., Milofsky, E. and Karnovsky, M. L.: Metabolic patterns in three types of phagocytizing cells. J. Cell Biol. 17: 487, 1963.

26. Barker, S.B. and Summerson, W. H.: The colormetric determination of lacetic acid in biological materials. J. Biol. Chem. 138: 535, 1941.

27. Myrvik, Q. N. and Evans, D.G.: Metabolic and immunological activities of alveolar macrophages. Environ. Health. 14: 92, 1967.

28. Wahler, B. E. and Wollenberg, A.: Zur Bestimmungdes orthophospats neben sancremolybdat - labelen phosphoroaureverbindungen. Biochem Z.329: 508, 1958.

29. Feinstein, R. N.: Perborate as substrate in a new assay of catalase. J. Biol. Chem. 180: 1197, 1949.

30. Chance, B. and Maehly, A.C.: Assay of catalases and peroxidase. In Methods in Enzymology. S. Colowick, editor. Academic Press, Inc. N.Y. 2:764, 1955.

31. Paul, B. and Sbarra, A.J.: The role of the phagocyte in host-parasite interactions. XIII. The direct quantitative estimations of H_2O_2 in phagocytizing cells. Biochem. Biophys. Acta. 156: 168, 1968.

32. Brandt, R. and Keston, A.S.: Synthesis of diacetyldichloro-fluorescin: A stable reagent for fluoremetric analysis. Anal. Biochem. 11:6, 1965.

33. Heise, E.R., Myrvik, Q.N. and Lealse, E.S.: Effects of Bacillus Calmette Guerin on the levels of acid phosphatase, lysozyme and cathepsin in rabbit alveolar macrophages. J. Immunol. 95: 125, 1965.

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References to Experimental Procedures And Methods

34. Hofstee, B.H.J.: Direct and continuous spectrophotometric assay of phosphomonesterases. Arch. Bioch. 51: 139, 1954.

35. Fishman, W.H., Springer, B. and Bruenetti, R.: Application of improved glucuronidase assay method to study of human blood B - glucuronidase. J. Biol. Chem. 173: 449, 1948.

36. Anson, M. L.: The estimations of pepsin, trypsin, papain and cathepsin with hemoglobin, J. Gen. Physiol. 22: 79, 1938.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Microbiology Research Laboratories are located as a complex in the Triboro Hospital at Queens Hospital Center, Jamaica, New York. They include: (a) an aerosol exposure laboratory, (b) a smoke-exposure laboratory, (c) individual laboratories for macrophage studies and microbiology, (d) 2 rooms for storage space and refrigerators. Animal quarters are provided in another area of the hospital. The entire laboratory area occupies approximately 700 square feet.

The equipment in this area includes a complete bacterial aerosol generating and exposure system with mixing chambers and decontaminating units, and an Anderson apparatus for measuring particle size of bacterial aerosols: A cigarette smoke generating apparatus and exposure chamber and a sequential sampler and gas liquid chromatography unit for determining concentration of the particulate and gas phase of cigarette smoke. Other major instrumentation present include the following: (a) standard microscopes, (b) 1 infusion pump, (c) a centrifuge, (d) 2 large refrigerators, (e) sonic dismembranator, (f) 2 water baths and shaker, (g) 1 freezer, (h) 2 incubators and 1 environmental chamber, (i) 4 vacuum pumps, (j) pH meter, (k) Beckman DU2 recording spectrophotometer, (l) spectronic 20 spectrophotometer, (m) Gilson respirometer, (n) a lyophilizer unit, (o) immuno and disc electrophoresis apparatus, (p) large autoclave, (q) analytical balance and (r) flash evaporator. There is also equipment for qualitative and quantitative bacteriology studies, tissue homogenation, administration of gas mixtures and animal surgery. A Revco deep freezer (-75°C) is also available. High performance scintillation counters capable of isotope work are present in the hospital and available for research use. In addition, a fully equipped laboratory capable of performing histological and electron microscopy studies are available to the Division of Microbiology.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

See Pages 6-13

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See Pages 14-15

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4.

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Joseph J. Guarneri, Ph.D. Principal Investigator	20 hrs/60 hrs	
Boris A. Shidlovsky, Ph.D.	7 hrs	
Research Associate, Ph.D. (to be recruited)	100%	
	Fringe Benefits	
Technical		
Research Technician (to be recruited)	100%	
	Sub-Total for A	

REDACTED

B. Consumable supplies (by major categories)

See page 4A for detailed list

Sub-Total for B 5,150

C. Other expenses (itemize)

Travel	900
Publications	100

Sub-Total for C 1,000

Running Total of A + B + C 35,485

D. Permanent equipment (itemize)

Sorvall RC 5 Automatic Superspeed
Refrigerated Centrifuge including heads ($4905)

Sub-Total for D 4,905

E. Indirect costs (15% of A+B+C) E 5,323

15. Estimated future requirements: Total request $45,712

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	5,200	1,000	1,000	5,683	$41,565
Year 3		5,200	1,000	500	6,063	$46,980

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B. Consumable Supplies

1.	Cigarettes for smoke studies	$1,000
2.	Mice Caesarian delivered	1,000
3.	Rabbits	1,200
4.	Immunodiffusion Plates, Anti SIgA and Anti Secretory Piece Sera, Radial Immunodiffusion kit and templates	200
5.	Chromatography columns with 4 way valve, accessories, and Reagents for Chromatography, Sephadex G 200, Acrylamide kit, DEAE	303
6.	Reagents and curvettes for enzyme studies and flasks and accessories for respiration studies	500
7.	Tissue culture glassware, tissue culture media and bacteriology media, and nebulizers.	547
8.	Isotopes uniformly labelled glucose - 1-C^{14} and glucose 6-C^{14}	300
9.	Petri dishes and plastic disposables	100
	Total	$5,150

D. Permanent Equipment (Justification)

1. The Sorval RC 5 Superspeed Refrigerated Centrifuge is needed for the alveolar lining material studies (ALM) proposed on pages 2A-9 and 2A-10 of grant request. The instrument and its rotors permit centrifugation up to 49,500 g with controlled temperature. The latter permits g forces and controlled conditions necessary for the isolation of ALM without the loss of biological activity.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Influence of Extended Exposure to Cigarette Smoke on Pulmonary Resistance to Infection as Related to Alveolar Macrophages and Mucociliary Function.	The Council for Tobacco Research, U.S.A. Grant Nos. 547C, 547 CR-1 and 547 CR-2	$65,152	7/1/71 to 6/30/75
Important Determinants of Pulmonary Resistance to Infection, Alcoholic Intoxications.	Long Island Jewish-Hillside Medical Center Grant No. 274	22,413	5/3/74 to 6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Joseph J. Guarneri, Ph.D.

Signature [redacted] Date 1/21/75

Telephone _____
Area Code Number Extension

Checks payable to

ng Island Jewish-Hillside Medical Center

Mailing address for checks

Mrs. Eva Meyer, Grant Manager
Long Island Jewish-Hillside Medical Center
New Hyde Park, New York 11040

Responsible officer of institution

Typed Name Harold L. Light

Title Deputy Director

Signature _____ Date 1/21/75

Telephone 212-343-6700 (Ext.2723)
Area Code Number Extension

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12 Biographical Sketch Joseph J. Guarneri, Ph.D. Principal Investigator

NAME: Joseph J. Guarneri, Ph.D.

ADDRESS: HOME: REDACTED

OFFICE: Long Island Jewish-Hillside Medical Center/Queens Hospital Center
82-68 164th St., Jamaica, New York 11432

TELEPHONE: REDACTED
HOME:
OFFICE: 212-990-2335

BIRTHDATE AND PLACE: REDACTED

CITIZENSHIP: REDACTED

MARITAL STATUS: REDACTED

SOCIAL SECURITY #: REDACTED

EDUCATION: REDACTED
- New York University; B.A. (Biology), 6/49
- Saint John's University; M.S. (Microbiology), 6/63
REDACTED
- Saint John's University; Ph.D. (Microbiology), 6/66

INTERNSHIP RESIDENCY: Not applicable.

MILITARY SERVICE: 1/49 - 3/46 - Sergeant, U.S. Army; Medical Corp., Camp Lee, Virginia

BOARD STATUS: American Society for Microbiology; Certification as Specialist in Public Health and Medical Laboratory Microbiology.
American Academy of Microbiology; Fellowship (Pending).

TYPE OF PRACTICE: Not applicable.

LICENSURE STATUS: Certificate of Qualification for Director of a Clinical Microbiology Laboratory, City of New York, Department of Health.

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ACADEMIC POSITIONS:

9/61 - 6/66 - Research Associate, Division of Respiratory Diseases, New Jersey College of Medicine and Dentistry.

6/66 - 6/68 - Instructor in Medicine, Department of Medicine, New Jersey College of Medicine and Dentistry, Jersey City, New Jersey.

7/68 - 1/72 Director, Pulmonary Aerobiology Research Laboratory, Division of Infectious Diseases, Department of Medicine, Saint Vincent Hospital, Worcester, Mass.

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ACADEMIC POSITIONS:

1/72	Attending Microbiologist, Long Island Jewish-Hillside Medical Center/Queens Hospital Center.
6/73	Associate Clinical Professor Pathology, SUNY at Stony Brook.
6/73	Coordinator Allied Health Sciences, Queens Hospital Center, Jamaica, New York.
9/73	Associate in Microbiology, St. John's University, Jamaica, New York.

MEMBERSHIP IN PROFESSIONAL SOCIETIES:

REDACTED

REDACTED

REDACTED

REDACTED

HONORS AND AWARDS:

Sigma XI, Saint John's University, 1963

3/68 to 5/69 - Public Health Service. H.I.H. Award #A.I. 08963-01.
Title: The Mechanism of Pulmonary Resistance to Infection. Principal Investigators: G.A. Laurenzi, M.D. Associate Director: J.J. Guarneri, Ph.D. - Amount: $ 67,769.

7/68 to 6/69 - The Council for Tobacco Research-U.S.A. Grant Award #547. Title: The Effect of Cigarette Smoke on the Nature and Function of Alveolar Macrophages. Principal Investigator: G.A. Laurenzi, M.D. Co-Investigator: J. J. Guarneri, Ph.D. - Amount $36,135.

9/68 to 6/71 - Saint Vincent Hospital Research Foundation. Title: The Role of the Alveolar Macrophage in Pulmonary Defense Against Inhaled Bacteria. Principal Investigator: J. J. Guarneri, Ph.D. Amount: $ 18,667.

6/69 to 5/72 - Public Health Service. H.I.A. Award #AI 08963-02. Title: The Mechanism of Pulmonary Defense Against Infection. Principal Investigator: G.A. Laurenzi, M.D. Associate Director: J.J. Guarneri, Ph.D. - Amount: $128,137.

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HONORS AND AWARDS:

7/69 - 6/71 - The Council for Tobacco Research - U.S.A. Grant Award #547BR1. Title: The Effect of Cigarette Smoke on the Immunological and Metabolic Function of Alveolar Macrophages. Principal Investigator: J.J. Guarneri, Ph.D. - Amount: $29,380.

7/71 - 6/72 - Saint Vincent Hospital Research Foundation. Title: The Influence of Bacterial Species on the Anti-bacterial Activity of Alveolar Macrophages. Principal Investigator: J. J. Guarneri, Ph.D. Amount $ 4,000.

7/71 - 6/75 - The Council for Tobacco Research - U.S.A. Grant Award # 547C. Title: The Influence of Extended Exposure to Cigarette Smoke on Pulmonary Resistance to Infection as Related to Alveolar Macrophage and Muco-ciliary Function. Principal Investigator: J.J. Guarneri, Ph.D. - Amount: $63,000.

5/74 - 6/75 - Long Island Jewish-Hillside Medical Center #274. Title: Important Determinants of Pulmonary Resistance to Infection, Alcoholic Intoxication. Principal Investigator: J. J. Guarneri, Ph.D. - Amount: $22,413.

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PUBLICATIONS: (Papers published or in press)

1. Laurenzi, G.A., Guarneri, J.J., Endriga, R.B. and Carey, J.P.: Clearance of Bacteria by the Lower Respiratory Tract. Science: 142: 1572-1573, 1963.

2. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B. "Important Determinants in Pulmonary Resistance to Bacterial Infections." In the Pathogenisis of Chronic Obstructive Broncho-Pulmonary Disease. In Mitchell R.S.: Progress in Research in Emphysema and Chronic Bronchitis, New York, S. Karger, 1965, p. 45-59.

3. Laurenzi, G.A., Guarneri, J. J. and Endriga, R.B.: Important Determinants in Pulmonary Resistance to Bacterial Infection. Medicinia Thoracalis 22: 48-59, 1965.

4. Laurenzi, G.A., and Guarneri, J.J.: A Study of the Mechanisms of Pulmonary Resistance to Infection: The Relationship of Bacterial Clearance to Ciliary and Alveolar Macrophage Function. Symposium on Structure, Function, and Measurement of Respiratory Cilia. Am. Rev. of Respiratory Dis. 93: 134-141, 1966.

5. Laurenzi, G.A., Yin, S., Collins, R., Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Current Research in Chronic Obstructive Lung Diseease. U.S. Public Health Service Publication No. 1787: 27-40, 1967.

6. Laurenzi, G.A., Yin, S., and Guarneri, J. J.: The Adverse Effect of Oxygen on Tracheal Mucus Flow. New England J. of Med. 279: 333 - 339, 1968.

7. Guarneri, J.J. and Laurenzi, G.A.: The Effect of Alcohol on the Mobilization of Alveolar Macrophages. J. of Lab. and Clinical Med. 72: 40-51, 1968.

8. Combs, T.J., Guarneri, J.J., and Pisano, M.A. The Effect of Sodium Chloride on the Lipid Contents and Fatty Acid Composition of Candida Albicans. Mycologia LX: 1232 - 1239, 1968.

9. Guarneri, J.J.: Clearance of Inhaled Bacteria from the Murine Respiratory Tract. In Developments In Industrial Microbiology. American Institute of Biological Sciences, Washington, D.C., Volume 15, 1974 (In Press).

10. Guarneri, J.J.: Influence of Acute Exposure to Cigarette Smoke on the Alveolar Macrophage System. J. Lab. Clin. Med., 1975. Submitted to CTR for publication support.

ABSTRACTS: (abstracts published and presented or read by title)

1a. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Bacterial Clearance from the Lung of Mice, Fed. Proc.: 22: 255, 1963.

2a. Laurenzi, G.A., Endriga, R.B., Guarneri, J.J. and Carey, J.P.: Important Determinants in Resistance to Pulmonary Infection. J. Clinical Invest. 949: 42, 1963.

3a. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Important Determinants in Pulmonary Resistance to Infection: Proceeding of Seventh Aspen Conference on Bronchitis and Pulmonary Emphysema. p. I-4, 1964.

4a. Laurenzi, G.A., Collins, B.J., Yin S and Guarneri, J.J.: The Adverse Effects of High Oxygen Breathing and Hypoxia. J. Clin Invest. 45: 1035, 1966.

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5a. Laurenzi, G.A., Yin, S., Collins, B. J. and Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Proceedings of the Tenth Aspen Conference. p. 100, 1966.

6a. Laurenzi, G.A., Collins, B.J., Yin, S. and Guarneri, J.J.: Adverse Effect of High Oxygen Breathing on Tracheobronchial Mucus Flow. Amer. Rev. Resp. Dis. Vol. 96: 152, 1967.

7a. Guarneri, J.J., Combs, T.J., and Pisano, M.A.: Lipid Components of Candida stellatoide Bacteriol Proc. p. 84, 1967.

8a. Guarneri, J.J.: Lipid Composition of Candida stellatoides. Disertation Abstracts 27: 3614-B, 1967.

9a. Guarneri, J.J. and Laurenzi, G.A.: The Mobilization of Alveolar Macrophages as a Pulmonary Defense Mechanism Against Inhaled Bacteria. Bacteriol Proc. p. 100, 1968.

10a. Laurenzi, G.A., Yin, S., Collins, B.J. and Guarneri, J.J.: Mucus Flow in the Mamallian Trachea. Public Health Service Publication No. 1787, p. 27, 1967.

11a. Combs, T.J., Guarneri, J.J. and Pisano, M.A.: Effect of Growth Conditions on the Fatty Acid Composition of Candida Albicans. The Third Symposium on Yeasts, Delft-Hague, The Netherlands, June 2-7, 1969.

12a. Guarneri, J.J. and Laurenzi, G.A.: The Effect of Cigarette Smoke on Alveolar Macrophage Numbers. The 148th Annual Meeting of the New York City Branch of the American Society for Microbiology, New York, N.Y., Feb. 25, 1971.

13a. Guarneri, J.J. and Laurenzi, G.A.: Influence of Cigarette Smoke on Pulmonary Defense Against Inhaled Bacteria. Alveolar Macrophage Numbers and Viability. The 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., May 26, 1972. (Abstract No. M 170).

14a. Guarneri, J.J. and Sierra, M.F.: Antibacterial Activity of Alveolar Macrophages Against Staphylococcus aureus. The 1974 Annual Meeting of The American Society for Microbiology, New York City Branch, Wagner College, Staten Island, New York, April 15, 1974.

15a. Guarneri, J.J.: Influence of In Vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. The 1974 Annual Meeting of The American Society for Microbiology, New York City Branch, Wagner College, Staten Island, New York, April 15, 1974.

16a. Guarneri, J.J.: Influence of Extended Exposure to Cigarette Smoke on Pulmonary Defense against Inhaled Bacteria. 74th Annual Meeting of The American Society for Microbiology, Chicago, Ill, May 12-17, 1974. (Abstract No. M355).

17a. Guarneri, J.J.: Clearance of Inhaled Bacteria from Murine Respiratory Tract. 25th Annual Meeting of the Society for Industrial Microbiology, Memphis, Tenn., Aug. 11 - 16, 1974, (Abstract).

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18a. Guarneri, J.J.: Influence of Acute Exposure to Cigarette Smoke on Pulmonary Defense Mechanisms. 14th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif. Sept. 11-13, 1974. (Abstract No. 181).

19a. Guarneri, J. and Goldstein, J.: A Study of the In-Vitro Interaction Between Alveolar Macrophages and Staphylococcus aureus. The 1975 Annual Meeting of The New York City Branch of the American Society for Microbiology, Wagner College, Staten Island, New York, January 14, 1975.

PRESENTATIONS: (papers given by invitation and thesis)

1p. Guarneri, J.J.: The Inhibition of Bacteria by Aconitic Acid. Master's Thesis, St. John's University, June 9, 1963.

2p. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Important Determinants in Pulmonary Resistance to Infection: Proceedings of Seventh Aspen Conference on Bronchitis and Pulmonary Emphysema, p. 1-4, 1964.

3p. Laurenzi, G.A., Yin, S., Collins, B.J. and Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Proceedings of the Tenth Aspen Conference, p. 100, 1966.

4p. Guarneri, J.J.: Lipid Composition of Candida stellatoidea. Ph.D. Thesis, St. John's University, June 12, 1966.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

R: REDACTED MATERIAL

Biographical Sketch - Boris A. Shidlovsky, Ph.D.

NAME: Boris A. Shidlovsky, Ph.D.

ADDRESS:
Home: REDACTED
Office: Long Island Jewish-Hillside Medical Center/ Queens Hospital Center
82-68 164 Street, Jamaica, New York 11432

TELEPHONE:
Home: REDACTED
Office: 212-990-2337

DATE OF BIRTH: REDACTED

MARITAL STATUS:

EDUCATION:
R - New York University B.A.
- St. John's University M.S.
- St. John's University Ph.D.

MILITARY SERVICE: 1942 - 1946 U.S. Army Medical Corps. senior non-commissioned officer.

LICENSURE STATUS: Certificate of Qualification for Director of a Clinical Microbiology Laboratory, City of New York, Department of Health.

PROFESSIONAL EXPERIENCE:

1951-1961	Div. of Surg. Res. Lab. Harlem Hospital, New York City, Bacteriologist-in-charge.
1961-1962	Microbiology Department, New York University Dental School, New York, Research Associate.
1962-1963	Misericordia Hospital, New York, Chief Bacteriologist
1963-1966	Morrisania Hospital (Montefiore-Morrisania Hospital Affil.), Chief Bacteriologist
1966-1969	Quinton Research Labs/Merck & Co., Inc. (Senior Research Microbiologist)
1969-1974	Associate Professor at Monmouth College, West Long Branch, New Jersey
1974	Assistant Attending Microbiologist, Long Island Jewish-Hillside Medical Center/ Queens Hospital Center.

MEMBERSHIPS IN PROFESSIONAL SOCIETIES: REDACTED

REDACTED

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R: REDACTED MATERIAL

MEMBERSHIPS IN PROFESSIONAL SOCIETIES:

REDACTED

RESEARCH INTERESTS: Antimicrobial agents and host defense mechanisms

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13. Publications Pertinent to Material Covered in Grant Proposal*:

1. Guarneri, J. J. and Laurenzi, G.A. The Effect of Cigarette Smoke on Alveolar Macrophage Numbers. The 148th Annual Meeting of the New York City Branch of the American Society for Microbiology, New York City, Feb. 25, 1971.

2. Guarneri, J.J. and Laurenzi, G.A. Influence of Cigarette Smoke on Pulmonary Defense Against Inhaled Bacteria: Alveolar Macrophage Numbers and Viability. The 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., May 26, 1972. (Abstract No. M 170).

3. Guarneri, J.J. and Sierra, M.F. Antibacterial Activity of Alveolar Macrophages Against Staphylococcus aureus. The 1974 Annual Meeting of the American Society for Microbiology, New York City Branch, New York, April 15, 1974.

4. Guarneri, J.J. Influence of In Vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. The 1974 Annual Meeting of the American Society for Microbiology, New York City Branch, New York, April 15, 1974.

5. Guarneri, J. J. Influence of Extended Exposure to Cigarette Smoke on Pulmonary Defense against Inhaled Bacteria. 74th Annual Meeting of the American Society for Microbiology, Chicago, Ill. May 12-17, 1974. (Abstract No. M 355).

6. Guarneri, J.J. Clearance of Inhaled Bacteria from the Murine Respirator Tract. The 25th Annual Meeting of the Society for Industrial Microbiology, Memphis, Tenn., Aug. 11-16, 1974.

7. Guarneri, J.J. Influence of Acute Exposure to Cigarette Smoke on Pulmonary Defense Mechanisms. The 14th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., Sept. 11-13, 1974. (Abstract No. 181).

8. Guarneri, J.J. Clearance of Inhaled Bacteria from the Murine Respirator Tract. In: Developments In Industrial Microbiology. American Institute of Biological Sciences, Washington, D.C., Volume 16, 1975 (In Press).

9. Guarneri, J.J. Influence of Acute Exposure to Cigarette Smoke on the Alveolar Macrophage System. Journal of Lab. and Clin. Med., 1975. Submitted to CTR for publication support.

10. Guarneri, J. J. and Goldstein, J. .A study of the In Vitro Interaction Between Alveolar Macrophages and Staphylococcus aureus. The 1975 Annual Meeting of the American Society for Microbiology, New York City Branch, New York. Jan. 14, 1975.

11. Guarneri, J.J. Influence of In Vivo Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. Submitted for presentation at: The 75th Annual Meeting of the American Society for Microbiology, New York, 1975.

12. Guarneri, J.J., Goldstein, J. and Shidlovsky, B. Effect of In Vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. Submitted for presentation at: The 75th Annual Meeting of The American Society for Microbiology, New York, 1975.

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* See Curriculum Vitae page 9 for a complete list of publications.
See Addendum II for copies of publications pertinent to material covered in grant proposal.

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

13. Publications Pertinent to Material Covered in Grant Proposal:

13. Guarneri,J.J. Influence of Acute Exposure to Cigarette Smoke on the Clearance of Bacteria by the Murine Respiratory Tract. (In Preparation).

14. Guarneri,J.J. Influence of Extended Exposure to Cigarette Smoke on the Clearance of Bacteria by the Murine Respiratory Tract. (In Preparation).

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Addendum II. Pertinent Personal Publications

Table of Contents

1. Laurenzi, G.A., Guarneri, J.J., Endriga, R.B. and Carey, J.P.: Clearance of Bacteria by the Lower Respiratory Tract. Science: 142: 1572-1573, 1963.

2. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Bacterial Clearance from the Lung of Mice, Fed. Proc.: 22: 255, 1963.

3. Laurenzi, G.A., Endriga, R.B., Guarneri, J.J. and Carey, J.P.: Important Determinants in Resistance to Pulmonary Infection. J. Clinical Invest. 949: 42, 1963.

4. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Important Determinants in Pulmonary Resistance to Infection: Proceeding of Seventh Aspen Conference on Bronchitis and Pulmonary Emphysema. p. 1-4, 1964. (Copy not available).

5. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B. "Important Determinants in Pulmonary Resistance to Bacterial Infections". In the Pathogenisis of Chronic Obstructive Broncho-Pulmonary Disease. In Mitchell, R.S.: Progress in Research in Emphysema and Chronic Bronchitis, New York, S. Karger, 1965, p. 45-59. (Copy not available).

6. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.R.: Important Determinants in Pulmonary Resistance to Bacterial Infection. Medicinia Thoracalis 22: 48-59, 1965.

7. Laurenzi, G.A., Collins, B.J., Yin S. and Guarneri, J.J.: The Adverse Effects of High Oxygen Breathing and Hypoxia. J. Clin Invest. 45: 1035, 1966.

8. Laurenzi, G.A., and Guarneri, J.J.: A Study of the Mechanisms of Pulmonary Resistance to Infection: The Relationship of Bacterial Clearance to Ciliary and Alveolar Macrophage Function. Symposium on Structure, Function, and Measurement of Respiratory Cilia. Am. Rev. of Respiratory Dis. 93: 134-141, 1966.

9. Laurenzi, G.A., Yin, S., Collins, B.J. and Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Proceedings of the Tenth Aspen Conference. p. 100, 1966. (Copy not available).

10. Laurenzi, G.A., Collins, B.J., Yin, S. and Guarneri, J.J.: Adverse Effect of High Oxygen Breathing on Tracheobronchial Mucus Flow. Amer. Rev. Resp. Dis. Vol. 96: 152, 1967.

11. Laurenzi, G.A., Yin, S., Collins, R., Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Current Research in Chronic Obstructive Lung Disease. U.S. Public Health Service Publication No. 1787: 27-40, 1967.

12. Laurenzi, G.A., Yin, S., Collins, B.J. and Guarneri, J.J.: Mucus Flow in the Mamallian Trachea. Public Health Service Publication No. 1787, p. 27, 1967. (Abstract). (Copy not available).

13. Guarneri, J.J. and Laurenzi, G.A.: The Mobilization of Alveolar Macrophages as a Pulmonary Defense Mechanism Against Inhaled Bacteria. Bacteriol Proc. p. 100, 1968.

14. Laurenzi, G.A., Yin, S., and Guarneri, J.J.: The Adverse Effect of Oxygen on Tracheal Mucus Flow. New England J. of Med. 279: 333 - 339, 1968.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Addendum II. Pertinent Personal Publications (cont'd.)

15. Guarneri, J.J. and Laurenzi, G.A.: The Effect of Alcohol on the Mobilization of Alveolar Macrophages. J. of Lab. and Clinical Med. 72: 40-51, 1968.

16. Guarneri, J.J. and Laurenzi, G.A.: The Effect of Cigarette Smoke on Alveolar Macrophage Numbers. The 148th Annual Meeting of the New York City Branch of the American Society for Microbiology, New York, N.Y., Feb. 25, 1971.

17. Guarneri, J.J. and Laurenzi, G.A.: Influence of Cigarette Smoke on Pulmonary Defense Against Inhaled Bacteria. Alveolar Macrophage Numbers and Viability. The 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., May 26, 1972. (Abstract No. M 170).

18. Guarneri, J.J. and Sierra, M.F.: Antibacterial Activity of Alveolar Macrophages Against Staphylococcus aureus. The 1974 Annual Meeting of The American Society for Microbiology, New York City Branch, Wagner College, Staten Island, New York, April 15, 1974.

19. Guarneri, J.J.: Influence of In Vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. The 1974 Annual Meeting of The American Society for Microbiology, New York City Branch, Wagner College, Staten Island, New York, April 15, 1974.

20. Guarneri, J.J.: Influence of Extended Exposure to Cigarette Smoke on Pulmonary Defense against Inhaled Bacteria. 74th Annual Meeting of The American Society for Microbiology, Chicago, Ill. May 12-17, 1974. (Abstract No. M 355)

21. Guarneri, J.J.: Clearance of Inhaled Bacteria from Murine Respiratory Tract. 25th Annual Meeting of the Society for Industrial Microbiology, Memphis, Tenn., Aug. 11-16, 1974. (Abstract)

22. Guarneri, J.J.: Clearance of Inhaled Bacteria from the Murine Respiratory Tract. In Developments in Industrial Microbiology. American Institute of Biological Sciences, Washington, D.C., Volume 16, 1974 (In Press).

23. Guarneri, J.J.: Influence of Acute Exposure to Cigarette Smoke on Pulmonary Defense Mechanisms. 14th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif. Sept. 11-13, 1974. (Abstract No. 181).

24. Guarneri, J. and Goldstein, J.: A Study of the In-Vitro Interaction Between Alveolar Macrophages and Staphylococcus aureus. The 1975 Annual Meeting of The New York City Branch of the American Society for Microbiology, Wagner College, Staten Island, New York, January 14, 1975.

25. Guarneri, J.J.: Influence of Acute Exposure to Cigarette Smoke on the Alveolar Macrophage System. J. Lab. Clin. Med., 1975. Submitted to CTR for publication support.

26. Guarneri, J.J.: Influence of In Vivo Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. Submitted for presentation at: The 75th Annual Meeting of the American Society for Microbiology, New York, 1975.

27. Guarneri, J.J., Goldstein, J. and Shidlovsky, B.: Effect of In Vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. Submitted for presentation at: The 75th Annual Meeting of the American Society for Microbiology, New York, 1975.

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#1025 - MITIMAN

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

February 4, 1975

Grant Application No. 1025

PULMONARY

To: The committee comprising Drs. Bing, Jacobson and Meier

Subject: Charles Mittman, M.D., City of Hope Medical Center
Duarte, CA.
New Application No. 1025
"Round Heart Disease in Turkeys: A Model of Antitrypsin Deficiency in Man."

History

Dr. Mittman has had previous CTR support (October 1, 1972 through September 30, 1973, and a continuation grant for the period October 1, 1973 through September 30, 1974) for the program "Hereditary Susceptibility to Branchitis-Emphysema."

The present application (No. 1025) was handled as an informal inquiry (Case No. 301): The Executive Committee voted to encourage formal application.

The study involves a collaborative effort with the group at the Kimron Veterinary Institute in Israel (see New Application No. 1026).

Request

Application No. 1025 requests $7,877 for the first year of a proposed three year program.

Documents Submitted (attached):

1. Application dated January 30, 1975 (9 pages, including CV's of Drs. Mittman and Lieberman).

2. Nine reprints (1 with acknowledgment to CTR).

DS:jmb
Enclosures

D.S.

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#1625

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
FEB 3 1975
ARCHIVE

Application for Research Grant
(Use extra pages as needed)

Date: 1/30/75

1. Principal Investigator (give title and degrees):
A. Charles Mittman, M.D., Director, Respiratory Disease Department
B. Jack Lieberman, M.D., Associate Director, Respiratory Disease Dept.

2. Institution & address:
City of Hope Medical Center
1500 East Duarte Road
Duarte CA 91010

3. Department(s) where research will be done or collaboration provided:
Respiratory Disease Department, City of Hope Medical Center, Duarte, California 91010

4. Short title of study:
Round Heart Disease in Turkeys: A model of Antitrypsin Deficiency in Man

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

Investigators at the Kimron Veterinary Institute of Tel Aviv University, Israel, have demonstrated that the pathology in turkey round heart disease (RHD) shares certain features with antitrypsin deficiency in man (see accompanying application). Specifically, birds who die with RHD have a deficiency of alpha$_1$-globulins and specific intracytoplasmic globules in their liver cells. Studies on material sent to our laboratory from Israel confirm that the histochemical properties of the globules and their location in rough endoplasmic reticulum are identical to that seen in human material. We are currently conducting studies on material from turkeys dying of RHD in California.

This proposal requests funds to permit continued collaboration between our laboratory and the investigators in Israel. Specifically, we will be responsible for the following aspects of the study:

1) Consult with the Israeli investigators and assist in establishing procedures for the electrophoretic differentiation of the normal and aberrant alpha$_1$ protease inhibitor.
2) Chemically characterize the inhibitor isolated from the liver of normal and deficient turkeys.
3) Carry out electron microscopic studies of tissues from diseased and normal turkeys.
4) Determine if the condition recognized as round heart disease in turkeys in the United States is the same as that observed in Israel.

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8. Brief statement of working hypothesis:

See application of Trainin, et al)

9. Details of experimental design and procedures (append extra pages as necessary)

For a number of years our laboratory has been studying various aspects of alpha$_1$-antitrypsin deficiency (AATD) in man. Progress in understanding the basic defect in this inherited condition would be greatly accelerated if an animal model of the deficiency were available. Turkey round heart disease (RHD) appears to be associated with the same hepatic abnormality seen in human antitrypsin deficiency. Accordingly, study of RHD may yield important insights as to the cellular defect which interferes with normal hepatic synthesis and/or release of antitrypsin. The group at the Kimron Veterinary Institute in Israel first recognized the similarity between RHD and AATD. They are ideally situated to carry out certain aspects of the proposed research. Specifically, they have ready access to large numbers of diseased and healthy birds, have the facilities needed to carry out the crucial breeding studies, and are in possession of most of the equipment and skills needed for this project. Our group will contribute its experience with the various electrophoretic techniques for identifying variant antitrypsin types and its familiarity with the methods for isolating and characterizing the protein in liver and serum. (Please see reprint, Matsubara, Yoshida and Lieberman. Proc Nat Acad Sci 71:3334, 1974.) The necessary electron microscopic studies of various tissues will be performed in our laboratory. (Please see reprint, Gordon, Dixon, Rogers, Mittman, and Lieberman. Human Path 3:361, 1972.) Finally, techniques similar to those outlined above will be applied to material from turkeys dying of RHD in the United States.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Biochemistry Laboratory of the Respiratory Disease Department, City of Hope Medical Center, occupies 1600 square feet and contains all the equipment needed to carry out the required electrophoretic and biochemical studies. Protein characterizations are conducted in collaboration with the Department of Biochemical Genetics at the City of Hope (Dr. A. Yoshida, Director). Electron microscopy is performed using the facilities of the Department of Pathology, Laboratory of Cytogenetics and Electron Microscopy (Dr. R. Teplitz, Director).

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

12. Biographical sketch

CURRICULUM VITAE - CHARLES MITTMAN, M.D.

Born: REDACTED REDACTED

REDACTED

Education:

University of Chicago
A.B. with Honors
B.S. with Honors REDACTED
M.D. with Honors

McCoy College of Johns Hopkins University
Elective courses in Mathematics
September 1962 to June 1964

University of Chicago
Committee on Mathematical Biology
July 1965 to June 1966

Training and Experience:

Rotating Internship, University of Chicago Clinics
July 1960 to June 1961

Junior Assistant Resident in Medicine,
University of Chicago Clinics
July 1961 to December 1961

Surgeon, USPHS, Gerontology Branch, National Heart Institute, Baltimore City Hospital
December 1961 to June 1964

Assistant Visiting Physician in Medicine,
Baltimore City Hospital
February 1962 to June 1964

Assistant in Medicine, Johns Hopkins University School of Medicine
July 1963 to June 1964

Assistant Resident in Medicine,
University of Chicago Clinics
July 1964 to June 1965

USPHS Post-doctoral Fellow, Department of Medicine,
University of Chicago
July 1965 to June 1966

Instructor, Department of Medicine,
University of Chicago
January 1966 to December 1966

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Director, Respiratory Disease Department,
City of Hope Medical Center, Duarte, CA
January 1967 to present

Assistant Clinical Professor of Medicine,
UCLA School of Medicine, Los Angeles, CA
June 1967 to June 1973

Associate Clinical Professor of Medicine,
UCLA School of Medicine, Los Angeles, CA
July 1973 to present

PUBLICATIONS

1. Mittman C: Summary of symposium on pulmonary emphysema and proteolysis. Amer Rev Resp Dis 105:430-448, 1972.

2. Mittman C, Barbela TV and Lieberman J: Antitrypsin deficiency and abnormal protease inhibitor phenotypes. Arch Envir Health 27: 201-206, 1973.

3. Mittman C and Lieberman J: Screening for Alpha$_1$-Antitrypsin deficiency. Israel J Med Sci 9:1311-1318, 1973.

4. Mittman C, Lieberman J and Rumsfeld J: Prevalence of abnormal protease inhibitor phenotypes in patients with chronic obstructive lung disease. Amer Rev Resp Dis 109:295-296, 1974.

5. Mittman C, Pedersen E, Barbela T and Lewis H: Prediction and potential prevention of industrial bronchitis. Amer J Med 57: 192-199, 1974.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

R: REDACTED MATERIAL

12. Biographical sketch

CURRICULUM VITAE - JACK LIEBERMAN, M.D.

Born: REDACTED
Marital Status: REDACTED

Education: University of California at Los Angeles REDACTED
B.A. with honors (Zoology)

Northwestern University - REDACTED
University of Southern California, Los Angeles REDACTED
M.D. R

Training and Experience:

Internship, Los Angeles County Harbor General Hospital, 1954-55.

Resident in Internal Medicine, Harbor General Hospital, 1955-58.

Clinical Assistant in Medicine, UCLA School of Medicine, 1957-58.

Research Fellow of the Long Beach Heart Association, Harbor General Hospital, 1958-60.

Clinical Instructor in Medicine, UCLA School of Medicine, 1958-62.

Attending Staff, Harbor General Hospital, 1960 to present

Clinical Investigator, Veterans Administration Hospital, Long Beach, 1960-63.

Assistant Clinical Professor in Medicine, UCLA School of Medicine, 1962-68.

Section Chief, Internal Medicine, Veterans Administration Hospital, Long Beach, CA. 1963-68.

Associate Clinical Professor in Medicine, UCLA School of Medicine, 1968-71.

Associate Director, Department of Respiratory Diseases, City of Hope Medical Center, Duarte, CA. 1968-

Director, Pulmonary Biochemistry Laboratory, City of Hope Medical Center, 1968-

Associate Clinical Professor in Medicine, University of California at Irvine School of Medicine, 1971-

Boards: Diplomate of American Board of Internal Medicine, 1962.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

PUBLICATIONS

1. Lieberman J and Mittman C: Dynamic response of alpha$_1$-antitrypsin variants to diethylstilbestrol. Amer J Human Genet 25:610-617, 1973.

2. Lieberman J: Plasma arginine esterase activity in cystic fibrosis. Amer Rev Resp Dis 109:399-400, 1974.

3. Lieberman J: Editorial: Emphysema, cirrhosis, and hepatoma with alpha$_1$-antitrypsin deficiency. Ann Int Med 81:850-852, 1974.

4. Lieberman J: Carboxypeptidase B-like activity and C3 in cystic fibrosis. Amer Rev Resp Dis 111:100-102, 1975.

5. Matsubara S, Yoshida A and Lieberman J: Material isolated from normal and variant human liver that immunologically crossreacts with alpha$_1$-antitrypsin. Proc Nat Acad Sci 71:3334-3337, 1974.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Charles Mittman, MD	10	-
Jack Lieberman, MD	10	-
Technician	25	2,500
Technical		
Sub-Total for A		2,500

B. Consumable supplies (by major categories)

Animals	50	
Reagents, antibody, etc.	100	
Sub-Total for B		150

C. Other expenses (itemize)

Electron microscopy on approximately 10 sets of tissues	2000	
Airfreight and shipping of samples	500	
Travel to Israel	1200	
Travel within the U.S.	500	
Sub-Total for C		4,200
Running Total of A + B + C		6650

D. Permanent equipment (itemize)

None

Sub-Total for D	
E	1,027
Total request	7,877

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	3,000	150	4,500	0	1,147	8,797
Year 3	3,500	200	4,500	0	1,230	9,130

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Dr. Mittman: Genetic and Environmental Influences on Lung Disease	Public Health Serv. HL12833	32,349	9/1/74 to 8/31/75
Dr. Lieberman: Alpha$_1$-Antitrypsin Deficiency in Pulmonary Emphysema	Public Health Service HL13398	41,516	1/1/75 to 12/31/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Dr. Mittman: Genetic and Environmental Influences on Lung Diseases	Renewal of Public Health Service HL12833	170,836	9/1/75 to 8/31/78

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Charles Mittman

Signature [signature] Date 1-20-75

Telephone 213 (Area Code) 3598111 (Number) 771 (Extension)

Checks payable to

City of Hope Medical Center

Mailing address for checks

1500 East Duarte Road

Duarte CA 91010

Responsible officer of institution

Typed Name Elihu King

Title Assistant Administrator

Signature [signature] Date 1-2[illegible]

Telephone 213 (Area Code) 359-8111 (Number) 709 (Extension)

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

#1026 - TRAININ

1003546356

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

February 4, 1975

Grant Application No. 1026

PULMONARY

To: The committee comprising Drs. Bing, Jacobson and Meier

Subject: Ze'ev Trainin, Dr. Med. Vet., Kimron Veterinary Institute, Israel

New Application No. 1026
"Round Heart Disease in Turkeys as an Animal Model for Alpha-1-Antitrypsin Deficiency in Man."

History

This application is the result of a collaborative effort planned with Dr. Charles Mittman: Dr. Mittman has submitted a separate proposal to CTR (see Grant Application No. 1025), for support of his part of the joint investigation.

Request

Application No. 1026 requests $31,175 for the first year of a three year program.

Documents Submitted (attached)

1. Application dated January 29, 1975 (14 pages including CV's of Drs. Trainin, Bogin, and Neumann (and Dan Ratter, M.Sc.).

Comment

I am enclosing two reprints for your information. These were submitted by Dr. Mittman in his original letter of inquiry.

DS:jmb
Enclosures

D.S.

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#1036

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

RECEIVED
FEB 3 1975

Application for Research Grant
(Use extra pages as needed)

Date: 1/29/75

1. Principal Investigator (give title and degrees):
   1) Ze'ev Trainin, Dr. Med. Vet. Head, Department of Immunology
   2) Eitan Bogin, Ph.D., Head, Department of Biochemistry
   3) Filip Neumann, Dr. Med. Vet. Ph.D., Department of Pathology

2. Institution & address:
   Kimron Veterinary Institute, affiliated with Tel-Aviv University, Bet Dagan, P. O. B. 12, Israel

3. Department(s) where research will be done or collaboration provided:
   Department of Immunology, Kimron Veterinary Institute
   " Biochemistry, " " "
   " Pathology, " " "

4. Short title of study:
   Round heart disease in turkeys as an animal model for alpha$_1$-antitrypsin deficiency in man.

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

In man, alpha$_1$-antitrypsin (alpha$_1$AT) deficiency is known to be hereditary and has been well documented. The accumulated data indicate that alpha$_1$AT deficiency leads to a liver cirrhosis and chronic obstructive lung disease (COLD). The absence of a suitable animal model has limited the extent of research carried out on the disease.

Round Heart disease (RHD) of turkeys seems to be the suitable animal model. In the last two years (1973-4) workers at the Departments of Pathology and Immunology have found similarities between RHD of turkeys and alpha$_1$AT deficiency in man.

Specific intracytoplasmic liver cell globules were found in RHD of turkeys. These globules are histologically and histochemically identical to those found in alpha$_1$AT deficiency in man. These globules contain alpha globulins as was demonstrated by immunofluorescent technique. Blood analyses of turkeys with RHD showed a reduction of total serum proteins and a characteristic alpha globulin deficiency. Furthermore, serum of diseased turkeys showed a deficiency in the antitryptic activity.

All these data suggest the similarity between RHD of turkeys and alpha$_1$AT deficiency in man.

This project seeks to prove the value of the turkey model by:

a. Hereditary studies - identification of the different genotypes in relation to the proteinase inhibitor.

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b. The physical and biochemical characterization of the proteinase inhibitor in turkeys' serum and its specificity towards different proteinases.

c. Showing relationship between alpha$_1$AT deficiency and the appearance of the syndrome.

d. The effect of different environmental conditions by exposure of the birds to different factors (temperature, pollution etc.)

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8. Brief statement of working hypothesis:

Round Heart Disease in turkey poults seems to be in individuals with low serum trypsin inhibition capacity (STIC) values. This may be caused either by defective inhibitor, by blocked release mechanism in the liver cells that produce the inhibitor or by a lower production of the inhibitor. The cause of the lower STIC is hereditary and the development of the RHD is conditioned by other factors such as temperature, air and water pollution, nutritional imbalance, administrated drugs or a combination of these all.

The etiology of RHD in turkeys shows a close similarity with the alpha$_1$AT deficiency and the development of obstructive lung emphysema in man. A clarification of the hereditary mechanism of RHD in turkey poults will possibly help in the understanding of alpha$_1$AT deficiency in man. Knowledge of proteinase inhibitors and their function will enable a better understanding of the causes and development of the diseases, their followup and possibly their treatments.

9. Details of experimental design and procedures (append extra pages as necessary)

A. A survey will be made in turkey flocks to study the frequency of RHD, relative frequency of different STIC levels and proteinemia. The survey will be carried out by:
- Examination of all dead poults
- Taking blood from a random sample from each flock, and testing for the following parameters:
  - Serum protein fractions by means of immunodiffusion tests
  - STIC levels
  - Total serum protein levels
  - Serum globulin levels

B. The electrophoretic pattern of turkey serum proteins will be studied in order to find the existing phenotypes. It is intended to investigate the possibility of establishing a reliable diagnostic test in order to identify poults with RHD before their death. The methods which will be used are immunoelectrophoresis, immunodiffusion and immunofluorescence.

C. An experimental flock of turkeys will be established in the institute farm from birds which will be collected from infected flocks. The birds will be grouped according to STIC levels and crosses will be made between difference groups determined by their alpha$_1$AT serum levels. Egg samples will be reserved from each cross and hatched. Serum types will be defined in the progeny of each cross to find the particular mode of inheritance of the different groups.

D. A followup of each group will consist of determining causes of death and pathological changes during their growth and development. Birds will be sacrificed and their liver, heart and lungs will be checked histologically and enzymatically. Blood will be taken from the birds and various serum enzyme levels will be determined in an attempt to monitor any internal changes.

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E. Poults of different phenotypes will be exposed to different stresses such as temperature extremes, nutritional imbalances, crowding and pollution in an attempt to find genetic and environmental interactions and factors influencing the development of RHD.

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F. The inhibitor alpha$_1$AT from turkeys' serum will be isolated and characterized physically and chemically (MW, PH, sub-units, terminal amino-acids, amino-acids composition and conjugated moiety such as carbohydrates). The specificity of the isolated inhibitor will be checked against different proteases especially those found in the heart and lungs.

G. Inhibitor from diseased turkey poults will be isolated from both serum and liver and be compared with the inhibitor isolated from normal poults; this will enable us to learn whether the problem is in the synthesis of the inhibitor or its release to the blood.

Time schedule of research program:

First year:
a. Survey of sample flocks
b. Study of normal STIC level in turkeys
c. Electrophoretic analysis of turkey serum
d. Isolation of proteinase inhibitor and characterization from turkey serum
e. Establishment of the experimental flock

Second year:
a. Analysis of crosses in the experimental flock
b. Histologic and enzymatic study of diseased poults
c. Isolation and characterization of the inhibitor in healthy and deficient turkeys

Third year: Study of environmental factors that influence the outbreak of disease.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The research will be conducted at Israel's Kimron Veterinary Institute, Bet Dagan. The research will be done in collaboration by three departments - Immunology, Biochemistry and Pathology. All equipment and facilities will be available for this research.

Some of the equipment available for the research are:

- Ultracentrifuge
- High speed refrigerated centrifuges
- Spectrophotometers
- Fraction collectors
- Sonicators
- Oxygenograph
- Equipment for chromatography
- " " electrophoresis
- Scintillation and strip counters
- Gas chromatography
- Atomic absorption spectrophotometer
- Microscopes (including fluorescence)
- Standard equipment for histological preparation

In addition there are facilities in the institute farm for keeping and maintaining the experimental flock.

Additional studies will be carried out in collaboration with investigators at the City of Hope Medical Center, Duarte, California. (Please see accompanying application for details)

11. Additional facilities required:

Spectrophotometer - VIS U.V. digital readout

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

[redacted]

12. Biographical sketch Ze'ev Trainin

Title: Head, Immunology Dept.

REDACTED

Place of birth: Affula, Israel

Education: M.D.V. University of Bern, Switzerland - 1963

Dr. Med. Vet. " " " " - 1966

Honors: A. Kimron Prize for research in bovine leukemia - 1969

A. Kimron Prize for research in Round Heart disease in turkeys - 1974

Major research interest: Immunological deficiencies in newborn and malignant processes

Research and/or professional experience:

Head, Dept. of Immunology, Kimron Veterinary Institute, Bet Dagan, Israel 1970-present

Head, Section of Immunology and Serology, Dept. of Bacteriology,
Kimron Veterinary Institute, Bet Dagan, Israel 1967-1969

Assistant Dept. of Microbiology, School of Veterinary Medicine, University of Bern.
1964-1966

Assistant Dept. of Pathology, School of Veterinary Medicine, University of Bern,
8/63 - 12/63

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

13. Publications Ze'ev Trainin

Trainin, Z., Meirom, R. Calf immunoglobulins and congenital malformation. Res. Vet. Sci. 15, 1 (1973).

Trainin, Z., Klopfer, U., Meidan, G. and Meirom, R. Levels of IgM and IgG in the serum of normal and leukemic calves. J. comp. Path. 83,115 (1973).

Meirom, R., Trainin, Z., Barnea, A., Neumann, F., Klopfer, U., Nobel, T.A., Disson, M.S. and Plener, O. Hypoproteinemia and alpha globulin deficiency in Round Heart disease of turkeys. Vet. Rec. 34, 262 (1974).

Neumann, F., Meirom, R., Nobel, T.A., Trainin, Z. and Klopfer, U. La maladie du coeur rond des dindons conception etiologigue actuelle. Cah. med. Vet. 43, 100 (1974).

Meirom, R., Trainin, Z. and Neumann, F. Alpha globulin intracytoplasmic hepatocyte globules in Round Heart disease in turkeys, visualized by immunofluorescent technique. Poultry Sci. (In press)

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

R: REDACTED MATERIAL

12. Biographical sketch Eitan Bogin

Title: Head, Department of Biochemistry

REDACTED

Place of birth: Tel-Aviv, Israel

Education: Hebrew University, Jerusalem, Israel. B.Sc. 1956-1959)

University of California, Los Angeles, U.S.A. Biochemistry M.Sc. (1964)

" Ph.D. (1965)

Major research interest: Clinical biochemistry and its uses in veterinary medicine. enzyme profiles of tissues and blood and changes due to diseases, nutritional status and effect of pollution.

Research and/or professional experience:

Head, Department of Biochemistry, Kimron Veterinary Institute, affiliated with Tel-Aviv University Medical School. 1970 - present

Senior Lecturer, Tel-Aviv University Medical School 1972 - present

Assistant professor, University of Southern California Medical School(1969-1970)

Research Assistant - University of California, Los Angeles (U.C.L.A.) (1960-1965)

Lecturer, Negev University, Beer Sheva A graduate course in clinical biochemistry (1971)

Senior Lecturer, Tel-Aviv University Medical School. Faculty of Veterinary Medicine. Two semesters course in:

Principles in general biochemistry and clinical biochemistry and its uses in veterinary medicine - (1971- today)

Visiting Associate Professor, Institute for Anatomy, Physiology and Hygiene. Rhine University, Bonn, Germany (1973)

Stipend 1961-1965. Isaac Loeb Foundation, Chicago, U.S.A.

Fellowship 1961-1965. University of California, Los Angeles, U.S.A.

Fellowship - Alexander von Humboldt

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

13. Publications Eitan Bogin

Bogin, E. and Ziv, G. Levels of LDH, GOT, ALP calcium, magnesium and phosphorus in normal and mastitic milk. Cornell Vet. 66, 666-676 (1973)

Bogin, E. and Hadani, A. Digestive enzymes in hard ticks. I. Proteolytic enzyme activity in the gut of Hyalomma excavatum female ticks. Z. Parasitenk. 41, 139-146 (1973).

Shlosberg, A., Bogin, E., Pipano, E. and Klinger, I. Serum enzyme levels in experimental theileriosis in calves. Refuah vet. 30, 24-27 (1973)

Bogin, E. The effect of deuterium oxide on oxidative phosphorylation of M.phlei. 1st Inter, Cong. of Bact.. 11p. 62 (1973)

Bogin, E. Studies on the effect of antibacterial compound from garlic on biological membranes. 9th Int. Cong. Biochem. p. 271 (1973).

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

R: REDACTED MATERIAL

12. Biographical sketch. Filip Neumann

Title : Running Director, Dept. of Pathology

REDACTED

Place of Birth: Logoj, Rumania

Education: D.M.V. Faculty of Veterinary Medicine, Bucharest (Rumania) - 1948

Dr. med. Vet. Faculty of Veterinary Medicin, Bucharest - 1949

Ph.D. Hadassah Medical School, Hebrew University, Jerusalem - 1968

Honors: A. Kimron Prize for research in bovine leucosis - 1968

A. Kimron Prize, for research in Round Heart disease in turkeys -1974

Major research interest: Pathology of circulatory system.

Research and/or professional experience:

Research Veterinary Pathologist, Kimron Veterinary Institute, Grade A - 1971-present

W.H.O. Senior research training grant (Munich, Antverpen, Cambridge) 1969-1970

Research Veterinary Pathologist, Kimron Veterinary Institute, grade B , 1967-1971.

Veterinary Pathologist, Kimron Veterinary Institute, 1959-1967

Lecturer, Department of Veterinary Pathology, Faculty of Veterinary Medicine, Arad (Rumania) 1954-1958

Assistant, Dept. of Histology, Faculty of Veterinary Medicine, Arad, Rumania 1949-1954.

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Source: https://www.industrydocuments.ucsf.edu/docs/mgdm0000

13. Publications Filip Neumann

Neumann, F. The relationship between vasa sanguinea vasorum and aortic degeneration in the domestic swine. Path. Biol. 20, 437-441 (1972).

Neumann, F. and Ungar, H. Spontaneous aortic rupture in turkeys and the vascularisation of the aortic wall. Canad. vet. J. 14, 136-138, 1973.

Neumann, F., Nobel, T.A. and Klopfer, U. Calcinosis in goats. J. comp. Path. 83, 343-350 (1973).

Neumann, F., Klopfer, U., Nobel, T.A., Dison, M.S. and Bendheim, U. Peculiar liver changes in Round Heart disease in turkeys. Vet. Rec. 93, 599-601 (1973).

Neumann, F., Rattner, D., Klopfer, U. and Nobel, T.A. The vasa vasorum patterns in the fetal aorta of sheep. Angiologica, 10. 270-273 (1973).

Neumann, F., Meirom, Ruth, Nobel, T.A., Trainin, Z. and Klopfer, U. Round Heart disease in turkeys. A new etiological approach. Cah. med. vet. 47, 100-105, (1974)

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

R: REDACTED MATERIAL

12. Biographical sketch Dan Rattner

REDACTED

Place of birth: Haifa, Israel

Education: B.Sc. Hebrew University, Jerusalem 1969

M.Sc. " " " 1971

Major research interest: genetics and biostatistics

Research and/or professional experience:

1970-71 Director of Animal Research farm Lahav affiliated with Kimron Veterinary Institute

1972 - present Assistant in Dept. of Genetics, School of Agronomy, Hebrew University, Jerusalem

13. Publications Dan Rattner

Rattner, D. A genetic comparison of quantitative traits in natural populations of the fungus Schysophyllum commune. M.Sc. thesis, Hebrew University, Jerusalem, 1971.

Neumann, F., Rattner, D., Klopfer, U. and Nobel, T.A. The vasa vasorum patterns in the fetal aorta of sheep. Angiologica 10, 270 (1973)

Rattner, D., Bedrak, E., Feinberg, J. and Francus, G. A note on Mastitis. Metritis an Agalactia syndrome in the sow.

1003546369

Francus, G. and Rattner, D. : On the relation between milk production and fertility in kibbutz diary cattle herds. J. Agric. Sci. (In press).

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Dr. Z. Trainin	20	
Dr. E. Bogin	20	
Dr. F. Neumann	20	REDACTED
D. Ratner	100	
Technical help (animals)	50	3000
Sub-total		$ 10500
Sub-Total for A		
B. Consumable supplies (by major categories):		
Animals (purchase & maintenance)		$ 5000
Chemical and glassware		$ 5000
Sub-Total for B		$ 10,000
C. Other expenses (itemize)		
Travel to U.S.		$ 3000
Travel inland		$ 1000
Sub-Total for C		$ 4000
Running Total of A + B + C		$24,500
D. Permanent equipment (itemize)		
Spectrophotometer		$ 3000
Sub-Total for D		$ 3000
E		3675
Total request		$31,175

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	12,000	4,000	0	4,200	32,200
Year 3		13,500	4,000	0	4,650	35,650

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Dr. Z. Trainin

Signature _____ Date 26.12.74

Telephone _____
Area Code Number Extension

Checks payable to

_____

Mailing address for checks

_____

_____

Responsible officer of institution

Typed Name Prof. A. Hadani

Title Director Kimron Veterinary Institute

Signature _____ Date 26.12.1974

Telephone Israel 03-940682
Area Code Number Extension

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Reprinted from THE VETERINARY RECORD, December 8th, 1973. Pp. 599-601

Peculiar Liver Changes in Round Heart Disease in Turkeys

F. NEUMANN, Dr.Med.Vet., Ph.D., U. KLOPFER, Dr.Med.Vet.
and T. A. NOBEL, Dr.Med.Vet., D.T.V.M.
Kimvol Veterinary Institute, Bet-Dagan, Israel

M. S. DISON, B.A., B.V.Sc.
District Poultry Laboratory, Hedera

U. BENDHEIM, Dr.Med.Vet.
Yavne Hatchery

Vet. Rec. (1973). **93**. *599-601*

SUMMARY.—Specific intracytoplasmic liver cell globules were described in round heart disease in turkeys. These globules are histologically similar to those found in alpha-1 antitrypsin deficiency, an inherited condition in man. A hypothesis on the resemblance between these two conditions is raised.

Introduction

ROUND heart disease in turkey was first described in Canada by Magwood and Bray (1962). Later the condition was reported from the United States (Meyhers & Heppner, 1964; Sautter, *et al.*, 1968; Jankus & Good, 1970; Noren, *et al.*, 1971). Laursen-Jones (1968) and Gallagher and Spence (1970) described briefly a similar condition in Great Britain and noticed the constant liver changes associated with an enlarged heart. The latter authors called this condition cardio-hepatic syndrome.

The condition was characterised clinically by marked depression and listlessness. The birds had ruffled feathers and were retarded in growth (Meyhers & Heppner, 1964). According to our experience, death occurred mainly in birds aged two to 10 weeks; the condition was also found in older turkeys, but without mortality. Male turkeys were predominantly affected. Hunsaker (1971) recorded a mortality rate of four to 10 per cent.

The aetiology of round heart disease in turkey is still unknown. Many factors have been considered including genetic predisposition (Meyhers & Heppner, 1964; Hunsaker, 1971), conditions of management and environmental agents (Jankus & Good, 1970). Noren, *et al.* (1971) suggested that the causal agent was a virus, which resembled morphologically the avian leukosis viruses.

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In the present study attention was given to the character of the liver changes and to a possible explanation concerning the aetiology of this condition.

Materials and Methods

Seventy-six Nicholas turkeys, aged two to 24 weeks, mostly males, with round heart disease, were necropsied and histological examination was performed.

For histological purposes the heart, liver, lungs and occasionally intestinal segments were harvested. The organs were fixed in neutral 10 per cent. formalin and 7μ paraffin sections were cut. The sections were stained by hematoxylin-eosin. Furthermore, the liver sections were stained by: Mallory's phosphotungstic acid hematoxylin (PTAH), periodic acid Schiff reaction (PAS), Hess and Hollander's toluidin blue stain, Steedman's alcian blue method. Frozen sections, 10μ in thickness were stained by Sudan IV.

Unstained DPX mounted deparaffinised liver sections were examined under ultra-violet light, using a HBO-200 mercury vapour bulb as a light source, dark field condenser and UG1, 2 mm. thick primary, Wratten 2A secondary filter system.

Results and Discussion

The macroscopic changes in the birds were identical to those described by the authors referred to above.

The most evident lesions were the enlargement of the heart (Fig. 1) as a result of the dilatation of both ventricles, with the right one more prominent (Fig. 2), in addition the liver was enlarged, with rounded borders (Fig. 1). The parenchyma was hardened and generally of a greyish-brown colour.

Microscopic examinations Microscopic lesions of the heart were generally not observed. In some turkeys, focal infiltration of mononuclear cells in the myocardium was found. The epicardium in some birds showed a fibrinous exudation with mononuclear cell infiltration. The lungs and the intestines were without pathological changes.

Liver The liver changes were of a degenerative type. Vacuolisation of the liver cells was a constant finding and these cells were occasionally filled with fat. Areas of focal necrosis, bile duct hyperplasia and nodular mononuclear cell infiltration were observed in most of the cases. In older birds, a hyperplastic fibrosis of the liver parenchyma was found. These liver changes closely resemble those previously described by Sautter, *et al.* (1968).

The most distinctive liver changes, in our material, were intracytoplasmic globules, which ranged from two to 14μ in diameter and one to three such globules were found in the affected liver cells. The globules showed a characteristic histological picture with various staining methods. They were anhystic, eosinophile, PAS positive (Fig. 3). PTAH stains the spherules selectively a bluish black and the larger globules were surrounded by a clear halo (Fig. 4). With alcian blue and toluidin blue the globules remained unstained. By ultraviolet light examination the spherules showed a blue white autofluorescence (Fig. 5).

These intracytoplasmic globules seem to be similar

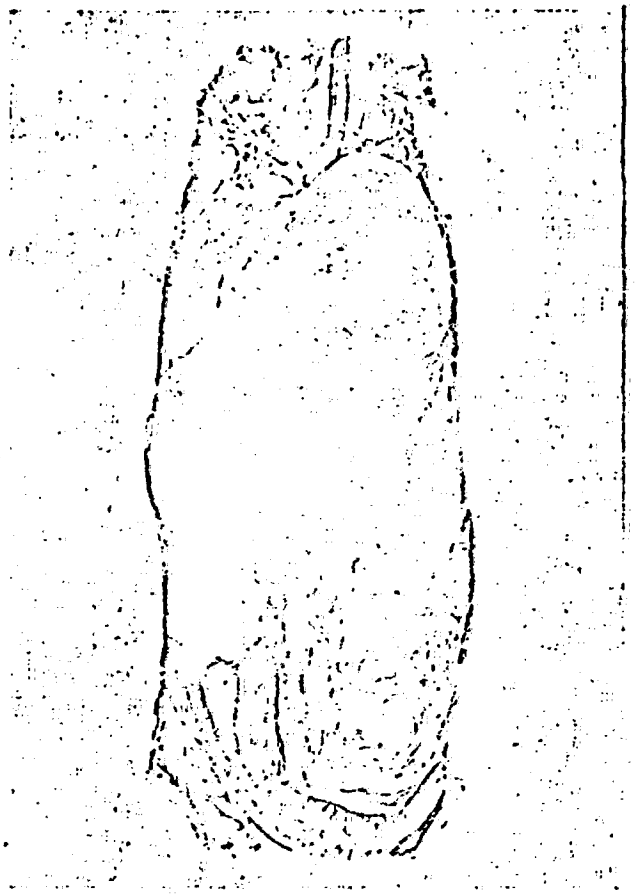

FIG. 1.—Five months old male turkey with an enlarged heart and liver.

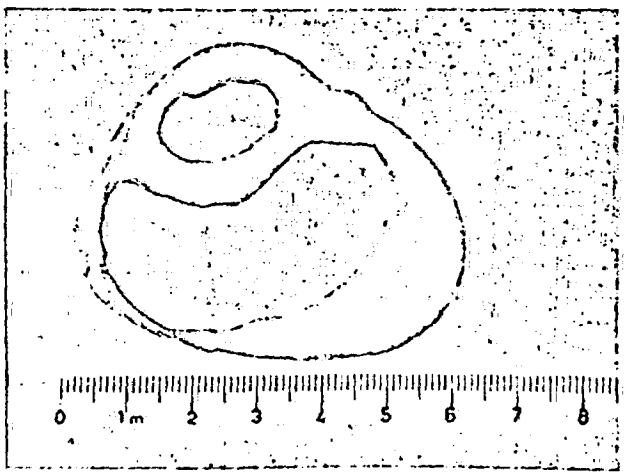

FIG .2—Five months old male turkey. Cross section through both ventricles.

to those found by DeLellis, *et al.* (1972), Gordon, *et al.* (1972), Lieberman, *et al.* (1972), Campra, *et al.* (1973) and Glasgow, *et al.* (1973) in alpha-1 antitrypsin deficiency in man, which is considered to be a hereditary condition. This similarity raises the supposition that round heart disease in turkey may be aetiologically related to the above condition in man. Further

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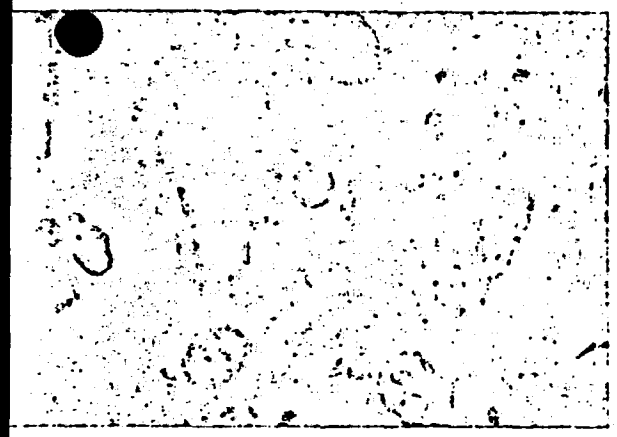

Fig. 3.—Seven weeks old male turkey. PAS positive globules in the cytoplasma of hepatocites. PAS stain x 630.

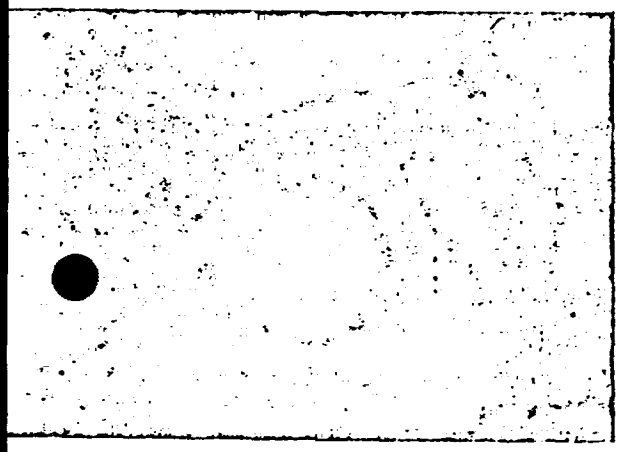

Fig. 4.—Two months old male turkey. Intracytoplasmic hepatocite globule with a bluish black centre and a clear rim. PTAH stain. x 1250.

upport for this hypothesis is the lack of alpha and eta globulins in the blood serum, of affected turkeys (Meirom & Trainin). In man the serum alpha globulins are 90 per cent. alpha-1 antitrypsin (Eriksson, 965).

Acknowledgment.—The authors are indebted to Professor P. P. Levine for the interpretation of some histological sections.

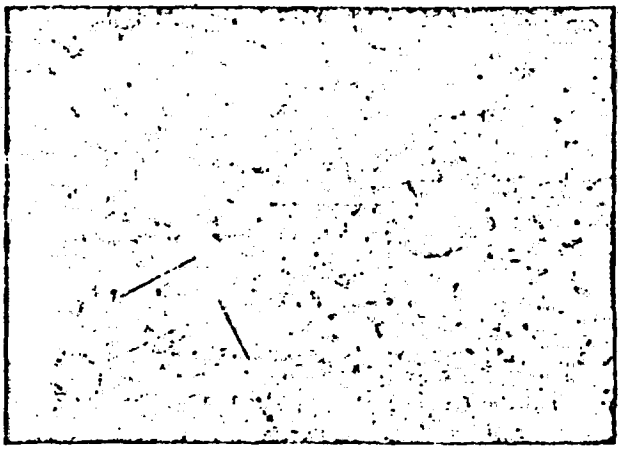

Fig. 5.—Two months old male turkey. Autofluorescence of the intracytoplasmic hepatocities globules. Unstained section, examined by U.V. light. x 625.

References

Campra, J. L., Craig, J. R., Peters, R. L., & Reynolds, T. B. (1973). *Ann. intern. Med.* 78. 233.

DeLellis, R. A., Balogh, K., Merk, F. B., & Chirife A. M. (1972). *Arch. Path.* 94. 308.

Eriksson, S. (1965). *Acta med. scandina.* 117. (Suppl. 432). 1.

Gallagher, J., & Spence, J. B. (1970). *Vet. Rec.* 86. 201.

Glasgow, J. F. T., Lynch, M. J., Hercz, A., Levison, H., & Sass-Kortsak, A. (1973). *Am. J. Med.* 54. 181.

Gordon, H. W., Dixon, J., Rogers, J. C., Mittman, C., & Lieberman, J. (1972). *Hum. Path.* 3. 361.

Hunsaker, W. G. (1971). *Poultry Sci.* 50. 1720.

Jankus, E. F., & Good, A. L. (1970). *Minnesota vet.* 10. 11.

Laursen-Jones, A. P. (1968). *Vet. Rec.* 83. 156.

Lieberman, J., Mittman, C., & Gordon, H. W. (1972). *Science.* 175. 63.

Magwood, S. E., & Bray, D. F. (1962). *Canad. J. comp. Med vet. Sci.* 26. 268.

Meirom, R., & Trainin, Z. In preparation.

Meyhers, P., & Heppner, R. G. (1964). *Feedstuffs.* 36. 56.

Noren, G. R., Staley, N. A., Jankus, E. F., & Stevenson, J. E. (1971). *Virchow Arch. path. Anat., Abt. A.* 352. 285.

Sautter, J. A., Newman, J. A., Kleven, S. H., & Larsen, C. T. (1968). *Avian Dis.* 12. 614.

Résumé

Des globules spécifiques intracytoplasmatiques dans les cellules du foie sont décrits dans la maladie du coeur rond des dindons. Ces globules sont similaires a ceux trouvés dans la deficience d'alpha-1 antitrypsine de l'homme. L'hypothèse de la resemblance des ce deux maladies est avancée.

Zusammenfassung

Spezifische kugelförmige intracytoplasmatische Leberzelleinschlüsse werden bei der Rundherzkrankheit von Puten beschrieben. Diese Körperchen ähneln denen die bei Alpha-1 Antitrypsindefizienz des Menschen gefunden wurden. Es wird die Hypothese von der Ähnlichkeit beider Krankheiten aufgeworfen.

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ORIGINAL PAPERS

Hypoproteinemia and alpha globulin deficiency in round heart disease of turkeys

RUTH MEIROM, MSc, Z. TRAININ, Dr med vet, ADA BARNEA, BSc, F. NEUMANN, Dr med vet, PhD, U. KLOPFER, Dr med vet, T. A. NOBEL, Dr med vet, DTVM, Kimron Veterinary Institute, Bet Degan, Israel
M. S. DIXON, BA, BVSc, District Poultry Laboratory, Hadera. O. PLESSER, DVM, District Poultry Laboratory, Jerusalem

Vet. Rec. (1974) 94, 262-264

SUMMARY.—Turkeys affected with round heart disease had a marked reduction in total serum protein. While the normal mean value was 3·8 ± 0·4g per 100 ml, the total serum protein value of the turkeys with round heart disease was 2·1 ± 0·7 g per 100 ml. The immunoelectrophoretic analyses in 87 per cent of the affected sera revealed a characteristic alpha globulin deficiency. The comparison between this phenomenon and alpha-1 antitrypsin deficiency in man is discussed.

Introduction

THE clinical and pathological aspects of round heart disease (RHD) in turkeys were reviewed by Neumann, *et al* (1973). The same authors described specific intracytoplasmic liver cell globules in the above condition, and emphasised the similarity between these globules and those found in alpha-1 antitrypsin deficiency in man, as described by: DeLellis, *et al* (1972); Gordon, *et al* (1972); Lieberman, *et al* (1972); Campra, *et al* (1973); Glasgow, *et al* (1973). This condition in man is considered to be hereditary. Because of similarity of hepatic lesions it was hypothesized that RHD of turkeys may be related etiologically to the above condition in man. Therefore, a study of the serum proteins of turkeys was undertaken to denote if changes similar to those found in man affected with alpha-1 antitrypsin deficiency, occur in turkeys with RHD.

Materials and methods

Complete necropsies were performed on 62 Nicholas turkeys with RHD, aged eight to 12 weeks, mostly males and on 123 healthy Nicholas turkeys aged 10 weeks. Forty-two of the RHD affected turkeys were dead when tested, all the other birds were slaughtered. For histological purposes representative pieces of liver were removed and fixed in 10 per cent neutral formalin. Periodic acid Schiff (PAS) reactions were completed on 7μ paraffin sections to detect the presence of PAS positive intracytoplasmic liver cell globules as described by Neumann, *et al* (1973) in RHD of turkeys.

An enlarged and dilated heart, as well as PAS positive globules in the liver cells were considered the criteria for a diagnosis of RHD and were used as a preliminary screening prior to the blood analyse.

Blood examinations

All blood samples were taken from the heart. The blood clots were removed on the same day as procured and the sera were stored at −20° C until tested.

Total protein estimation of serum was carried out by Biuret's method as described by Fey, *et al* (1964).

Antisera Anti-fowl and anti-turkey serum were produced by injecting rabbits with 0·1 ml of fowl serum and turkey serum respectively, with complete Freund adjuvant in the footpad. Boosters of 0·1 ml of serum were given intramuscularly three times at weekly intervals. The animals were bled a week after the last booster and the anti-fowl rabbit sera and anti-turkey rabbit sera were stored at −20° C until used.

Immunoelectrophoresis was carried out for all the tested sera as described by Trainin (1969). The antisera used were anti-fowl and anti-turkey rabbit sera.

Negative immunoelectrophoresis To determine proteins which might be lacking in the serum of affected turkeys, the following procedure was used. The electrophoresis was carried out with normal control serum, but before adding antiserum, 0·1 ml of the tested serum was put in the trough for five minutes and the antiserum was added as in regular immunoelectrophoresis. The antibodies which were not absorbed by the tested serum could react with the normal serum. Thus the precipitating lines represent only the proteins which are absent in the tested serum.

Results

Total serum protein in normal turkeys and with RHD

The sera of 47 healthy turkeys aged 10 weeks and the sera of 25 turkeys affected with RHD of the same age were tested in order to determine the total serum protein level. The mean value of the normal turkeys was 3·8 ± 0·4 (s.d.) g per 100 ml, while that of the affected ones was 2·1 ± 0·7 (s.d.) g per 100 ml. The variance of the affected birds was higher significantly ($p < 0.01$) than that of the healthy ones, as some of the affected turkeys had serum protein levels approximating that of the healthy ones. The means were compared accordingly, by the method suggested by Cochran and Cox (1957) and were found to differ significantly ($p < 0.01$). Hypoproteinemia in the

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serum of most of the affected turkeys could be seen clearly in the immunoelectrophoresis (Fig. 1).

Immunoelectrophoretic analyses

Immunoelectrophoretic tests of the sera of the affected turkeys (RHD) revealed a partial reduction of almost all serum proteins and a complete lack of one to two precipitating lines in the alpha globulin line (Figs 1 and 2), sometimes associated with the lack of the beta globulin line (Fig 2). One more precipitating line in the alpha region could be seen by the negative immunoelectrophoresis of the same sera. This line normally occluded by the albumin.

All 20 (100 per cent) slaughtered birds which were affected lacked alpha globulins in their sera. From 42 affected turkeys which were dead, 34 (81 per cent) also lacked the above mentioned globulin. In eight turkeys of the last group alpha globulins were present in their sera. Alpha globulin deficiency could be observed also in those affected birds which had normal total serum protein values.

In all, 54 affected turkeys (RHD) out of 62 (87 per cent) had an alpha globulin deficiency, as determined in the direct or negative immunoelectrophoretic tests. This deficiency was not observed in 123 healthy turkeys.

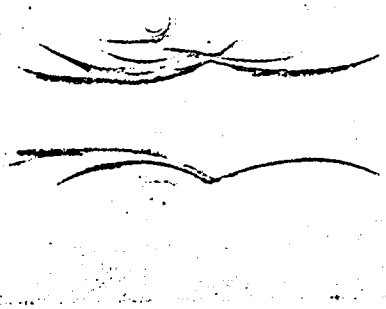

FIG 1: Immunoelectrophoretic analysis of turkey's sera. *Above*: Serum of 10 weeks old healthy turkey. *Below*: Serum of 10 weeks old turkey with Round Heart Disease. *Trough*: Rabbit anti turkey serum.

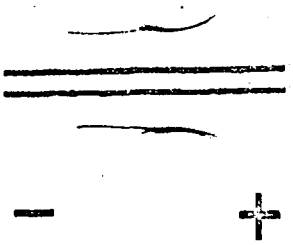

FIG 2: Negative immunoelectrophoretic analysis of turkey's sera. *Above and below*: Serum of 10 weeks old healthy turkey. *Trough*: Rabbit anti turkey serum absorbed with serum of 10 weeks old turkey affected with Round Heart Disease.

Comparative studies between serum and liver changes

To assess the relationship between a deficiency of alpha globulins and the concomitant present of cytoplasmic liver cell globules, a comparative study was completed. Forty-five RHD positive birds out of 62 revealed typical liver changes and an alpha globulin deficiency. In the remaining 17 cases either serum or liver changes occurred alone. Eight of nine birds with alpha globulin deficiency and without the intracytoplasmic liver cell globules were slaughtered.

Discussion

The total protein levels in the sera of the 10-week-old healthy turkeys are similar to those found by Bierer (1969). According to Clarkson (1966) and Bierer (1969) there are no marked differences among the protein levels of six to 12-week-old turkeys. The mean level of the serum protein of the RHD affected turkeys is significantly reduced, approximately 50 per cent. However, the variation in the levels among the affected turkeys is wide and some of the birds have protein values in the normal range. The possibility exists that the general reduction of the serum protein results from hepatic damage.

Round heart disease appears to be characterised by an alpha globulin deficiency. Such an alteration was not observed among the 123 healthy turkeys, but was seen in 87 per cent of the RHD affected turkeys. The fact that this alteration occurred also in turkeys with total protein levels in the normal range, suggests that an alpha globulin deficiency may be independent of the total protein reduction.

The comparative studies between typical serum and liver changes suggested that alpha globulin deficiency might occur before the intracytoplasmic liver cell globules are visible. The fact that these globules were not found in eight of 20 slaughtered turkeys with RHD, but were present in all dead birds, is similar to the observations of Gordon, *et al* (1972) in man. More intracytoplasmic liver cell globules were found in material obtained at autopsy than from hepatic biopsies obtained from individuals acutely ill.

Although, the difference between the percentage of slaughtered and dead affected birds having alpha globulin deficiency is not significant, it appears that serum values of liver of slaughtered birds are more representative than those of dead birds. The hypothesis that the proteins which are absent from the serum are produced, but blocked in the liver, as in alpha-1 antitrypsin deficiency in man (Gordon, *et al*, 1972) needs further investigation. In the event that the hypothesis will be proven, the possibility exists that the autolysis of liver cells after death may release these proteins to the serum and thus cause false "negative" reactions. This study has shown that immunoelectrophoretic analyses and especially the negative immunoelectrophoretic method can be used diagnostically in the detection of RHD affected turkeys. The alpha globulin which is normally invisible because it is masked by the albumin fraction resembles that of alpha-1 antitrypsin in man, in the immunoelectrophoretic picture (Erikson, 1965).

The specific identification of the deficient alpha

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globulin in the sera of RHD affected turkeys and the relationship, if any, to antitrypsin, needs further investigations.

Acknowledgments.—The authors are indebted to Mr D. Rattner for the statistical evaluations and to Professor J. Simon for revising the manuscript.

REFERENCES

BIERER, B. V. (1969). *Poultry Sci.* **48**, 1208.
CAMPRA, J. L., CRAIG, J. R., PETERS, R. L., & REYNOLDS, T. B. (1973). *Ann. intern. Med.* **78**, 233.
CLARKSON, M. J. (1966). *J. comp. Path.* **76**, 387.
COCHRAN, W. G., & COX, G. M. (1957). *Experimental Designs* (2nd edition). John Wiley and Sons, Inc., New York.
DELELLIS, R. A., BALOGH, K., MERK, F. B., & CHRIST, A. M. (1972). *Arch. Path.* **94**, 308.
ERIKSON, S. (1965). *Acta med. scandina.* **117**, (Suppl. 432), 1-80.
FEY, A., NICOLET, J., FELTENBERG, R., & MARGANDANT, A. (1964). *Zbl. Vet. Med. Reihe B.* **11**, 584.
GLASGOW, J. F. T., LINCH, M. J., HERCZ, A., LEVISON, H., & SASS-KORTSAK, A. (1973). *Am. J. Med.* **54**, 181.
GORDON, H. W., DIXON, JR, ROGERS, J. C., MELTMAN, C., & LIEBERMAN, J. (1972). *Hum. Path.* **3**, 361.
LIEBERMAN, J., MELTMAN, C., & GORDON, H. W. (1972). *Science*, **175**, 63.
NEUMANN, F., KLOPFER, U., NOBEL, T. A., DIXON, M. S., & BENDHEIM, U. (1973). *Vet. Rec.* **93**, 599.
ERASMUS, Z. (1969). *Am. J. vet. Res.* **30**, 1475.

RÉSUMÉ. Les dindons affectés avec la Maladie du Coeur Rond, demontrent une réduction importante de la proteine totale du serum. Tandis que, les valeurs moyens normaux sont de 3·8 ± 0·4 g pour 100 ml la proteine totale du serum chez les dindons affectee de la Maladie du Coeur Rond est de 2·1 ± 0·7 g pour 100 ml. Les analyses immunoelectrophoretiques montrent dans 87 pour cent des sera provenant des dindons atteints, une déficience characteristique des alpha globulins. La comparaison entre ce phénoméne et la alpha-1 antitrypsine déficience de l'homme est discutée.

ZUSAMMENFASSUNG. Die von Rundherzkrankheit befallenen Puten zeigen eine bedeutende Verringerung des totalen Proteins im Blutserum auf. Während die normalen Mittelwerte 3·8 ± 0·4 g pro 100 ml betragen, ist der totale Proteinwert des Blutserums bei mit Rundherzkrankheit befallenen Puten 2·1 ± 0·7 g pro 100 ml. Die immunoelektrophoretischen Analysen zeigen in 87 prozent der von kranken Puten untersuchten Sera einen charakteristischen Ausfall der Alpha Globuline. Der Vergleich zwischen diesem Phänomen und dem Alpha-1 Antitrypsinausfallides Menschen wird besprochen.

Ovine paralysis associated with spinal lesions of toxoplasmosis

B. A. McERLEAN, MVM, MRCVS, Department of Veterinary Medicine, Pharmacology and Food Hygiene, University College Dublin, Ireland

Vet. Rec. (1974), **94**, 264-266

SUMMARY.—Two Cheviot sheep, six months of age, developed a progressive paralysis of the limbs shortly after purchase. They had a persistent fever and elevated leucocyte counts. Extensive perivascular cuffing was found in the histological sections of the spinal cord and several *Toxoplasma* pseudocysts were seen.

Introduction

WHILE toxoplasmosis has been detected in many species of animals often in a latent form, it is of particular significance in sheep as in this species it is responsible for outbreaks of abortion and perinatal mortality. Crowley (1964) in Ireland described one outbreak where elevated serum titres were obtained following abortions. Apart from abortions and perinatal mortality there are very few reported cases of other clinical forms of toxoplasmosis in the adult sheep.

Olafson and Monlux (1942) in the United States, described a case in a ewe in which there were nervous signs, difficulty in moving, paresis and dyspnoea. Histological examination revealed an acute diffuse non-suppurative encephalomyelitis and *Toxoplasma* cysts were found in the brain. Wickham and Carne (1950) reported a similar case in Australia. Cole, *et al* (1954) in the U.S.A., recorded the deaths of five ewes and three lambs with signs of disease in the respiratory and central nervous systems. The only case recorded in the United Kingdom involved a two-year-old ewe in which encephalitic symptoms were seen (Zlotnik, 1959).

The purpose of this report is to record the first outbreak of clinical toxoplasmosis in adult sheep in Ireland.

Clinical report

A farmer living in the Dublin Mountains bought 40 six-month-old Cheviot sheep from a neighbour early in October, 1971. Two days later one sheep was unable to rise but got up when helped. A few days later it was unable to stand. Ten days later a second sheep began to sway on the hindquarters. On October 27, 1971 (Day 0) these two affected sheep were brought to the University College Dublin veterinary hospital. The first affected sheep (A) was paralysed in all four limbs. The second sheep (B) was able to stand but swayed unsteadily on the hind legs and resembled a case of sway-back. Both sheep were bright and showed no encephalitic symptoms. They had good appetites and ruminated frequently. Both animals had temperatures between 104·5 to 105·0 F until they were killed. Several blood samples were taken and the findings are indicated in Table 1. Radiographs were taken of the dorsal and lumbar spinal regions of each sheep. These failed to reveal any lesions. Each sheep received 2·5 ml of tetracycline* daily, commencing on Day 2. Sheep A received [illegible] injections and was killed on Day 6. Sheep B received several injections; however, it gradually

* Rexocin, Hoechst Pharmaceuticals

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

January 22, 1975

Grant Application No. 862A

PULMONARY

To: The committee comprising Drs. Bing, Liebow and Sommers

Subject: James Travis, Ph.D., University of Georgia
First continuation application No. 862A
"Biochemistry of Chronic Obstructive Lung Disease"

History

A three year program was approved by the SAB in March 1972 at a level of approximately $22,000. The third and final year (Grant No. 862R2) was supported at a level of $23,793.

The enclosed request is for a continuation of the work.

Request:

Application No. 862A requests $45,081 for the first year of a three year proposal.

Documents submitted (attached)

1. Application dated January 15, 1975 (13 pages).
2. Final progress report covering period July 1, 1972 - December 31, 1974 (7 pages).
3. C.V.'s of Drs. Travis, Matheson and Johnson.

D.S.

DS:wg
Encl.

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#6677

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JAN 20 1975

Application for Research Grant.
(Use extra pages as needed)

Date: Jan. 15/75

1. Principal Investigator (give title and degrees):

James Travis, Ph.D, Associate Professor

2. Institution & address:

University of Georgia
Athens, Georgia 30602

3. Department(s) where research will be done or collaboration provided:

Department of Biochemistry

4. Short title of study:

Biochemistry of Chronic Obstructive Lung Disease

5. Proposed starting date: July 1, 1975
6. Estimated time to complete: Three years
7. Brief description of specific research aims:

(see attached sheet)

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7. SPECIFIC RESEARCH AIMS

The process of pancreatic secretion of proteolytic enzymes is under double control in that these enzymes are a) all produced as inactive zymogens which cannot, therefore, degrade pancreatic tissue and b) they are secreted together with large quantities of a pancreatic protease inhibitor (Kazal secretory inhibitor). Thus, proteolysis is limited to the gut area. However, during acute attacks of pancreatitis, proteolysis is no longer controlled and not only are zymogens activated but also the secretory inhibitor is rapidly destroyed.

The proposed project essentially attempts to determine whether the parallel regulation of proteolysis in tissue is under control by serum factors. Particularly, we are asking the importance of α-1-PI, since it represents over 90% of the total inhibitory activity of serum or plasma. Because α-1-PI deficiency correlates strongly with the development of emphysema such a hypothesis is tempting. However, more questions have really been raised than have been answered. For example, does α-1-PI exert any control over major proteolytic reactions, such as occur in the coagulation and fibrinolytic systems? Can proteases secreted from phagocytizing cells actually overwhelm the normal regulatory system and degrade normal tissue? Are these proteolytic enzymes secreted in response to particular stimuli which do not come under the control of α-1-PI and therefore can degrade important organ tissue in individuals with normal levels of α-1-PI? It is believed that the experimental protocol suggested below will clarify the role of α-1-PI in regulating proteolysis. However, it is also probable that the results obtained will still leave us with more questions to ask than those which we have answered.

The objectives of this proposal are to determine the roles of α-1 proteinase inhibitor (α-1-antitrypsin), granulocytic elastase, and other proteases from granulocytes and macrophages, in tissue proteolysis. In particular, we wish to determine what structural differences account for the inability of α-1-PI type ZZ (and also type MZ) individuals to secrete sufficient amounts of inhibitor to combat proteolysis. We would also like to know the mechanism by which the inhibitor normally functions in order to possibly develop synthetic inhibitors. Finally, we would like to determine how the granulocytic and macrophage proteases function in order to again possibly develop better synthetic inhibitors to these enzymes. In this respect we hope to extend studies of this system to an investigation of the development of emphysema in individuals with normal α-1 PI levels. Thus, in individuals who inhale large quantities of particulate matter (i.e. cigarette smoke), are macrophage proteases being released in quantities large enough to overwhelm the normal defense mechanism or does the macrophage actually produce proteases which can digest lung tissue but which are not inhibitable by α-1-PI?

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8. Brief statement of working hypothesis:

It is now believed that the destruction of lung tissue, in type ZZ α-1-PI individuals (and to a lesser extent in type MZ) is due to unrestricted proteolysis by phagocytizing cells due to the absence of α-1-PI. It is suggested that the development of emphysema in individuals with normal α-1-PI may be due to the fact that either the defense mechanism against proteolysis is overwhelmed or that proteases, not inactivated by α-1-PI but capable of degrading lung tissue, are released.

9. Details of experimental design and procedures (append extra pages as necessary)

(see attached pages)

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9. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES

a) BACKGROUND

Human α-1-proteinase inhibitor (α-1-PI)[1], more commonly called α-1-antitrypsin, is a glycoprotein which has been shown to be responsible for approximately 90% of the trypsin inhibitory capacity of plasma (Heimburger et al., 1971). The broad specificity and high concentration of this protein in plasma and tissue fluids reflect its important role in preventing tissue proteolysis. The high incidence of phagocyte mediated proteolysis of lung tissue observed in some α-1-PI deficient individuals correlates with this function (Laurell and Eriksson, 1963; Fagerhol, 1972). This has resulted in an increased interest in both the properties and function of this protein.

During the past several years many laboratories have published methods for the isolation of human α-1-PI in various states of purification (Moll et al., 1958; Bundy and Mehl, 1959; Schultze et al., 1962; Shamash and Rimon, 1966; Liener et al., 1973; Murthy and Hercz, 1973; Crawford, 1973). Unfortunately, none of the procedures used are desirable because the isolated inhibitor is obtained either in low yield or in a partially inactive form.

As to the actual pathogen in the development of emphysema, it is believed to be proteolytic in nature, based on results such as those obtained by Mass et al., (1972) in which proteases were inhaled as an aerosol and produced emphysema - like lesions in the lung. The type of enzyme suspected is elastolytic in nature and its source is probably granulocytes and/or macrophages. The former type of cell has already been shown to be sequestered in lung bases and these cells can damage the pulmonary capillary membrane (Wilson et al., 1971).

An elastase preparation has been isolated from granulocytes by Janoff and Basch (1971) and, more recently, by Ohlsson (1974). Unfortunately, the purification procedures utilized were not easily followed or resulted in poor yield. At least three forms of elastase were identified, probably resulting from proteolytic cleavage either by autolysis or by other enzymes present during the purification steps.

Recent work on the mechanism of action of α-1-PI has been reported by Moroi and Yamasaki (1974). The results closely parallel those which are given in the current progress report. However, their α-1-PI is not homogenous and they assume 1:1 binding in the interpretation of the data. Nevertheless, the paper is an excellent beginning in understanding α-1-PI inhibition mechanisms.

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REFERENCES

Bundy, H. F., and Mehl, J. W. (1959), *J. Biol. Chem.* *234*, 1124.

Crawford, I. P. (1973), *Arch. Biochem. Biophys.* *156*, 215.

Fagerhol, M. K. (1972), *in* Pulmonary Emphysema and Proteolysis, Academic Press, New York, pp. 123-131.

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Heimburger, N., Haupt, H., and Schwick, H. (1971), *in* Proceedings of the 1st International Research Conference on Proteinase Inhibitors (Munich, November, 1970), Fritz, H., and Tschesche, H., eds., Walter de Gruyter, New York, pp. 1-21.

Janoff, A. and Basch, R. S. (1972), *Amer. Rev. Resp. Dis. 106*, 384.

Laurell, C. B., and Eriksson, S. (1963), *Scand. J. Clin. Lab. Invest. 15*, 132.

Liener, I. E., Garrison, O. R., and Pravda, Z. (1973), *Biochem. Biophys. Res. Commun. 51*, 436.

Mass, B., Ikeda, T., Meranze, D. R., Weinbaum, G., and Kimbel, P. (1972) *Amer. Rev. Resp. Dis. 106*, 384.

Moll, F. C., Sunden, S. F., and Brown, J. R. (1958), *J. Biol. Chem. 233*, 121.

Moroi, M. and Yamasaki, M. (1974) *Biochim. Biophys. Acta 359*, 130.

Murthy, R. J., and Hercz, A. (1973), *FEBS Letters 32*, 243.

Ohlsson, K. and Ohlsson, I. (1974), *Eur. J. Biochem. 42*, 519.

Schultze, H. E., Heide, K., and Haupt, H. (1962), *Klin. Wschr. 40*, 427.

Shamash, Y., and Rimon, A. (1966), *Biochim. Biophys. Acta 121*, 35.

Wilson, H. W., Ratliff, N. B., Mikat, E., Hackel, D. B., Young, W. G., and Graham, T. C. (1970), *in* 13th Aspen Conference on Respiratory Disease and Emphysema (Abstr. Chest 59) 36 S(1971).

b) SPECIFIC AIMS

During the next three years we hope to do the following: 1) Determine at a molecular level, differences between α-1-PI (MM) and α-1-PI (ZZ), 2) outline the mechanism by which α-1-PI inactivates proteolytic activity, particularly granulocytic elastase, 3) begin an investigation of other proteases, such as those in alveolar macrophages, which might be involved in degradation of alveoli and concomitant development of emphysema.

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c) METHODS OF PROCEDURE

The discernment of functional differences between α-1-PI (MM) and α-1-PI (ZZ) and the development of a possible mechanism by which α-1-PI inhibits proteolytic activity are overlapping problems. Our method of investigation suggests that of primary importance would be the elucidation of the complete structure, protein and carbohydrate side chain included, of the normal or "wild type" protein (type MM).

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Preliminary evidence suggests that major differences do occur in the carbohydrate composition of type MM and type ZZ proteins. However, the protein portion of both molecules may also have minor but important differences. Furthermore, if we are to attempt to establish the inhibition mechanism by which α-1-PI operates, it will be necessary to know something about its primary structure. For example, is there sequence homology between this serum inhibitor and that known to occur in other plant and animal proteinase inhibitors? Do the disulfide bond loops contain the active site of the inhibitor as is known to occur in other well-characterized proteins with this function? What are the cleavage points, if any, when trypsin, chymotrypsin, elastase and other proteinases complex with the inhibitor? We already know (see Progress Report-Part 2a) that α-1-PI does form a complex of MW 78,000 with one mole of trypsin or chymotrypsin and that further addition of protease causes formation of a 60,000 and 48,000 fragment with concomitant disappearance of the 78,000 MW species. How does this occur? What fragments are broken off and where do they come from? Are there two separate active sites on the inhibitor, one of which is not exposed until the 78,000 MW complex is formed? Why does elastase form a complex of MW ≈ 95,000? We feel that structural analysis will aid in elucidating this information. Furthermore, the results may give us some insight as to possible procedures for synthesizing low molecular weight inhibitors which are non-toxic and non-antigenic but whose function is similar to α-1-PI.

We propose to utilize α-1-PI (MM) purified in our laboratory for sequential analysis. We have already begun this study and have cleaved the reduced carboxymethylated protein at methionine residues with cyanogen bromide. We have separated the fragments by gel filtration and ion-exchange chromatography. We intend to sequence the peptides using a Beckman Model 800 sequenator. Where necessary, tryptic and chymotryptic cleavage will also be performed to provide overlapping peptides.

These experiments have now provided us with a C^{14} labelled peptide containing the cysteine residues. We feel, since we have no sequenator at present, that this is the most important part of the protein portion of α-1-PI which we should be examining since it may be at or near the inhibitory site(s). Dr. U. S. Seal, V. A. Hospital, Minneapolis, Minn., has identified the first nine residues in the intact protein for us, using a sequenator, as being Glu-asp-pro-glu-gly-asp-ala-ala-asp. Further analysis cannot be done for us because of reagent costs and a backlog of other samples in Dr. Seal's laboratory.

Carbohydrate side chain analysis of α-1-PI (MM) and α-1-PI (ZZ) is being performed with the assistance of Dr. J. Mendicino, a faculty member in our department who is well-recognized for his work with carbohydrates and glycoproteins. We will use inhibitor isolated by our standard procedure and prepare pronase digests of the two proteins. The glycopeptides will have been purified by ion-exchange chromatography. Each will be sequenced by addition of specific glycosidases followed by quantitative analysis of the sugar or sugar derivatives released. We are also using carbohydrate-containing cyanogen bromide fragments in this study. Our results with the type MM protein indicate to date the release of sialic acid (3 residues), N-acetyl hexosamine, and mannose. Quantitation is, however, not yet complete. We do not plan to use the ZZ protein, which we are accumulating, until the MM structure is conclusively established. This applies not only to carbohydrate analysis but also to peptide fingerprints in establishing any differences in the primary protein structure of the two molecules.

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Our further investigations into the mechanism of action of α-1-PI include formation of stable complexes with various proteases, followed by dissociation with hydrazine. The modified inhibitor and other fragments will then be analyzed to see exactly what peptide bonds have been cleaved and where, in the sequence, this is occurring. We hope to establish whether limited proteolysis releases a biologically active fragment with inhibitory properties. This has already been well documented in coagulation processes, complement activations, and zymogen activation. In particular, we would hope to detect two inhibitory sites on -1-PI for elastase, which are both accessible without peptide bond cleavage, to explain the formation of the stable complex of MW ≈ 95,000 which we have detected by SDS gel electrophoresis.

Finally, with regard to α-1-PI, we wish to know whether this protein does combine with thrombin or plasmin. Preliminary experiments indicate that incubation of -1-PI with thrombin does prolong clotting times. Hence, it may regulate the clotting process to some extent. We hope to detect stable complex formation by SDS gel electrophoresis of mixtures of the purified inhibitor and proteases.

Our investigations on the role of elastolytic enzymes in tissue proteolysis and, in particular, with regard to the development of emphysema will continue. To date, no one has attempted to show that granulocytic elastase is truly digesting lung alveoli. We will make antibodies to our human granulocytic elastase and attempt to demonstrate directly or indirectly by fluorescein isothiocyanate treatment the binding of the antibody to elastase in lung tissue. We intend to use normal and diseased tissue in these experiments and, in particular, material obtained from type ZZ individuals who have expired due to emphysema. This latter material is being provided by several hospitals including Mayo Clinic, Johns Hopkins Hospital, and Washington University School of Medicine. Some samples have already been obtained from Dr. R. C. Roberts, Marshfield Clinic. We are currently trying to establish a working relationship with Medical College of Georgia, Augusta, Ga.

Should the results be positive for enzyme localization in the lung, we intend to set up a radioimmunoassay for elastase in serum using I^{125} labelled enzyme. We hope to detect differences in circulating elastase levels which we will try to correlate with various disease states and with α-1-PI phenotypes.

Finally, we plan to examine the properties of granulocytic elastase in some detail in terms of its relationship to elastase(s) from other cell lines (see below) and with regard to its inhibition by other naturally occurring inhibitors. Again, it may be possible, through a detailed delineation of the physical, chemical, and biological properties of the molecule, to develop synthetic inhibitors to this enzyme which might prove useful both therapeutically and for prevention of unrestricted proteolysis.

Since we believe that macrophages and, probably, other cell types as well produce proteolytic enzymes in response to various stimuli we wish to initiate a study of these enzymes. We had planned to harvest such cells by bronchial lavage but this does not seem likely to result in the production of enough material to make enzyme isolation practical. We recently noted a new technique [PNAS 71, 3598 (1974)] for the elicitation of collagenase in the culture fluid of endotoxin-activated peritoneal macrophages. Although we will use macrophages obtained by

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bronchial lavage in our preliminary studies, we feel that it would be extremely useful to attempt to culture human alveolar macrophages and to try and stimulate these cells to produce proteases in the culture medium. The media could be harvested and utilized as a source of starting material for enzyme isolation.

Whether we use cells of media for enzyme isolation, we plan to purify as many of the major proteolytic enzymes present and determine whether they are inhibitable by α-1-PI. In particular, we will examine elastolytic proteases. We hope by these methods to determine exactly the role of macrophage proteases in the destruction of lung tissue.

d) SIGNIFICANCE

The proposed research should yield information as to the nature of the defect(s) in α-1-PI synthesis in type ZZ α-1-PI phenotypes. It will allow for the development of routine assays for elastase activity in serum as a possible clinical tool for diagnosis of potential emphysema patients. Finally, the actual significance of macrophages in the lung in terms of their role in lung destruction will be clarified. In particular, this should shed new light on the reasons for the development of emphysema in individuals with normal α-1-PI levels.

It should be noted that, as in the past, a fundamental rule in this laboratory is to work only with highly purified proteins. Crude systems only result in ambiguous results. We feel our approach, strictly at the molecular level, is the only reasonable methodology for truly understanding the roles of α-1-PI, elastase(s), granulocytes, and macrophages in the development of chronic obstructive lung disease.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location): The P.I. has a well-equiped laboratory with 1500 sq. ft. of space in the Department of Biochemistry in the Graduate Studies Research Center.

Major Pieces of Equipment directly available include the following:
Beckman Model 120C Amino Acid Analyzer (2)
Zeiss Model PMQ Spectrophotometer
Beckman Model E Analytical Ultracentrifuge
Fraction Collectors (4)
Radiometer pH Meter
Gilson High Voltage Electrophoresis (2)
Mettler Analytical Balance
Mettler Top Loading Balance
Radiometer Conductivity Meter
Sorvall Model RC2B Centrifuge
Table Top Centrifuge (2)

Also available in the department are:
Mass Spectrometers (2)
Novo Computers (2)
Scintillation Counters (4)
Cary Spectrophotometers (3)
Beckman Model L Ultracentrifuge (3)

A complete animal quarters for the preparation of anti-sera is also available.

11. Additional facilities required:

(see attached sheet)

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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11. ADDITIONAL FACILITIES REQUIRED:

Of paramount importance to this project is the purchase of a Beckman Sequenator. The P.I. has sequenced proteins of low molecular weight (6,000) by classical procedures and personally realizes the man-hours involved with a molecule as large as α-1-PI (53,000). There is no doubt that this is an awesome problem to tackle. Nevertheless, it must be accomplished if the true function of α-1-PI is to be realized. A sequenator can ultimately reduce the sequencing process to a realistic time, probably about one year, to complete the structure. Without it, sequencing by hand will probably take three years not only because of the manual manipulations involved but also because about ten to twenty times more protein will be required.

The P.I. realizes that a logical question to be asked is: Who else will use this instrument? What other proteins will be sequenced? In our own laboratory we would also plan to sequence human trypsin and human granulocytic elastase as both are unusual proteases, the former being important, in pancreatic function and the latter, probably, abnormal lung function. We feel that structural analysis will help us to determine why human pancreatic enzymes are so poorly inhibited by naturally occurring inhibitors. It goes without saying that the structure of granulocytic elastase will be very important if we are to later develop synthetic inhibitors against this enzyme. Our long range plans are to examine mast cell proteases and their role in the inflammatory process. Also, little is known of the function of α-1-antichymotrypsin. We hope to purify several of these proteins and probably determine their covalent structure in order to discern the mechanisms by which both the proteases and inhibitor function.

There are many other faculty members who would avail themselves of a sequenator were one purchased. A list of the faculty members and their interests is briefly described:

Dr. R. Lovins - hapten binding sites on antibodies
Dr. J. Mendicino - carbohydrate and protein structure of carcinoembryogenic antigen.
Dr. W. L. Williams - proteases (acrosin) and protease inhibitors in fertilization
Dr. M. J. Cormier - enzymes involved in vision and bioluminescence
Dr. H. D. Peck - bacterial electron-transfer proteins
Dr. J. Brewer - structure and function of enolase
Dr. L. Ljungdahl - structural differences between thermophilic and mesophilic enzymes with similar biological activities
Dr. F. Inman - structure of immunoglobulins

The diversity of interests by the above faculty members insures that good use would be made of a sequenator. In particular, it should be pointed out that Dr. Lovins has had considerable experience with sequenators and interfacing them with mass spectrometers for rapid analysis of protein sequences. NSF has recently allotted funds to him as a contribution to the purchase of a sequenator. The current purchase price is about $48,000. Should funds be allotted by The Council for Tobacco Research - U.S.A., the purchase of this instrument would be made from $12,000 from Dr. Lovins, through NSF, $12,000 through this grant from The Council for Tobacco Research - U.S.A., $11,000 from the University of Georgia equipment fund, and the remainder from the Department of Biochemistry.

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
James Travis, Associate Professor	50%	REDACTED
D. Johnson, Research Associate	50%	REDACTED
N. Matheson, Research Associate	100%	REDACTED
K. Beatty, Graduate Student	50%	REDACTED
Fringe Benefits		REDACTED
Technical		
J. Bowen, Research Technician	50%	REDACTED
Secretarial help	P.T.	REDACTED
Dishwasher (Student)	P.T.	REDACTED
Fringe Benefits		REDACTED
Sub-Total for A		REDACTED

B. Consumable supplies (by major categories)	
Glassware	$2,000
Chemical	2,000
Biological Materials (Blood, macrophages, etc.)	1,000
Sub-Total for B	$5,000

C. Other expenses (itemize)	
Travel (Foreign: $700; Domestic: $300)	$1,000
Publication Costs	800
Sub-Total for C	$1,800
Running Total of A + B + C	$28,070

D. Permanent equipment (itemize)	
Beckman Model 800 Sequenator	12,000
Digital pH Meter	800
Sub-Total for D	$12,800
E	4,211
Total request	$45,081

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	6,000	2,500	10,000	4,575	45,075
Year 3	R	6,000	2,500	3,000	5,175	42,675

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BUDGET JUSTIFICATION (General)

The justification for $12,000 towards purchase of a sequenator is presented in section 11. During the second year we anticipate the possible need for equipment for tissue culture in order to cultivate alveolar macrophages. Should this not be necessary it will, of course be dropped from the budget. Otherwise, justification will be made in the renewal application for the second year. In the third year a sharp increase in salary is requested to cover the summer salary of the P.I. who will no longer be supported by a Research Career Development Award at that time, his five year fellowship having been completed on June 30, 1977.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Biochemistry of Chronic Obstructive Lung Disease	CTR - 862R2	66,626	7/1/72 - 6/30/75
Proteolytic Enzymes of the Blood and Pancreas	NIH-HL-14478	123,000	1/1/72 - 12/31/76
Proteolytic Enzymes of the Blood and Pancreas	NIH Career Development Award 1K04 HL-70264		7/1/72 - 6/30/77

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name James Travis

Signature [signature] Date Jan. 15/75

Telephone REDACTED (Area Code / Number / Extension)

Checks payable to

The University of Georgia

Mailing address for checks

Vice President for Business & Finance
The University of Georgia
Athens, Georgia 30602

Responsible officer of institution:

Typed Name Robert C. Anderson

Title Vice-President for Research

Signature [signature] Date Jan. 15/75

Telephone REDACTED (Area Code / Number / Extension)

CD HRC

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R: REDACTED MATERIAL

CURRICULUM VITAE

NAME: James Travis

REDACTED

RANK: Associate Professor of Biochemistry

EDUCATION:

Year	Degree	Institution
1958	B.S.	University of Manitoba
1960	M.S.	University of Manitoba
1964	Ph.D.	University of Minnesota

TITLE OF MASTERS THESIS: "Separation and Quantitation of Riboflavin and its Derivatives in Mixtures."

TITLE OF DOCTORAL DISSERTATION: "Physico Chemical Properties of Porcine Trypsin." Advisor: Professor I. Eliener

EXPERIENCE:

Vitamin research - University of Manitoba, 1958-60, under supervision of Mr. A. D. Robinson.

Enzyme structure and function - University of Minnesota, 1960-64, under Dr. I. E. Liener.

Enzyme structure and function - Johns Hopkins University, 1964-66, under Dr. W. D. McElroy.

Enzyme structure and function - University of Maryland, 1966-67, as Assistant Professor.

Enzyme structure and function - University of Georgia, 1967-July, 1973, Assistant Professor, July 1972-present, Associate Professor.

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PUBLICATIONS:

1. J. Travis and A. D. Robinson. The Separation and Estimation of Riboflavin and Its Derivatives in Mixtures. Canadian J. Biochem. Physiol. 40, 1251-1252 (1962).

2. J. Travis and I. E. Liener. The Crystallization of Pork Trypsin. Arch. Biochem. Biophys. 97, 218-219 (1962).

3. J. Travis and I. E. Liener. The Crystallization and Partial Characterization of Porcine Trypsin. J. Biol. Chem. 240, 1962-19 (1965).

4. J. Travis and I. E. Liener. The Sequence of Amino Acids in the Vicinity of the Active Serine Residue of Porcine Trypsin. J. Biol. Chem. 240, 1967-1973 (1965).

5. J. Travis and W. D. McElroy. Isolation and Sequence of an Essential Sulfhydryl Peptide at the Active Site of Firefly Luciferase. Biochemistry 5, 2170-2176 (1966).

6. W. D. McElroy, M. DeLuca and J. Travis. Molecular Uniformity in Biological Catalysis. Science 157, 150-160 (1967).

7. J. Travis. The Specificity of Porcine Trypsin. Biochem. Biophys. Res. Comm. 29, 294-297 (1967).

8. J. Travis. Studies on the Active Site of Sheep Trypsin. Biochem. Biophys. Res. Comm. 30, 730-734 (1968).

9. J. Travis. Enzyme Active Sites. Atlas of Protein Sequence and Structure, pp. 57-62. Eck and Dayhoff (1967-68).

10. E. J. Laishley, J. Travis and H. D. Peck. Amino Acid Composition of Ferredoxin and Rubredoxin from Desulfovibrio gigas. J. Bact. 98, 302-303 (1969).

11. J. Travis and R. C. Roberts. Human Trypsin. Isolation and Physical-Chemical Characterization. Biochemistry 8, 2884-2889 (1969).

12. B. J. Gates and J. Travis. Isolation and Comparative Properties of Shrimp Trypsin. Biochemistry 8, 4483-4489 (1969).

13. M. H. Coan and J. Travis. Comparative Biochemistry of Proteases from a Coelenterate. J. Comp. Biochem. Physiol. 32, 127-139 (1970).

14. J. M. Brewer, T. Fairwell, J. Travis and R. E. Lovins. An Investigation of the Subunit Structure of Yeast Enolase. Biochemistry 9, 1011-1016 (1970).

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15. S. S. Lee, J. Travis and C. C. Black, Jr. Characterization of Ferredoxin from Nutsedge, Cyperus rotundus L., and Other Species with a High Photosynthetic Capacity. Arch. Biochem. Biophys. 141, 676-689 (1970).

16. M. S. Blum, J. B. Byrd, J. Travis, J. F. Watkins II and F. R. Gehlbach. Chemistry of the Cloacal Sac Secretion of the Blind Snake Leptotyphlops dulcis. J. Comp. Biochem. Physiol. 38B, 103-107 (1971).

17. M. H. Coan, R. C. Roberts and J. Travis. Human Pancreatic Enzymes. Isolation and Properties of a Major Form of Chymotrypsin. Biochemistry 10, 2711-2717 (1971).

18. M. H. Coan and J. Travis. Interaction of Human Pancreatic Proteases with Naturally Occurring Proteinase Inhibitors. In: Proceedings of the (First) International Research Conference on Proteinase Inhibitors, pp. 294-298. de Gruyter, Berlin, 1971.

19. J. Travis and M. H. Coan. Specificity of Inhibitors for Human Proteases. In: Proceedings of the International Symposium on Proteolysis and Pulmonary Emphysema. Academic Press, New York, N.Y., 1972.

20. D. J. Newman, J. Travis and H. D. Peck. Partial Sequence of a New Type Ferredoxin from a Sulfate Reducing Bacterium. In: Proceedings of the Tenth International Congress of Microbiology, p. 40, 1970.

21. J. Travis. Human Pancreatic Enzymes: Interaction of Human Trypsin with Chicken Ovomucoid. Biochem. Biophys. Res. Comm. 44, 793-796 (1971).

22. J. Travis, D. J. Newman, J. LeGall and H. D. Peck, Jr. The Amino Acid Sequence of Ferredoxin from the Sulfate Reducing Bacterium Desulfovibrio gigas. Biochem. Biophys. Res. Comm. 45, 452-458 (1971).

23. M. H. Coan and J. Travis. Human Pancreatic Enzymes: Properties of Two Minor Forms of Chymotrypsin. Biochim. Biophys. Acta 268, 207-211 (1972).

24. J. Travis. Human Pancreatic Enzymes: Amino Acid Sequence of the A-Chain of Human Chymotrypsin II. Biochem. Biophys. Res. Comm. 48, 1111-1116 (1972).

25. P. A. Mallory and J. Travis. Human Pancreatic Enzymes. Characterization of Anionic Human Trypsin. Biochemistry 12, 2847-2851 (1973).

26. B. J. Gates and J. Travis. Purification and Characterization of Carboxypeptidases A and B from the White Shrimp (Penaeus setiferus). Biochemistry 12, 1867-1874 (1973).

27. B. J. Gates and J. Travis. Amino Acid Sequence in the Vicinity of the Reactive Serine Residue in Shrimp Trypsin. Biochim. Biophys. Acta 310, 137-141 (1973).

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

28. S.-K. Oh, J. Travis and J. Brewer. Cysteines in Yeast Enolase. Biochim. Biophys. Acta 310, 421-429 (1973).

29. L. J. D. Zaneveld, G. F. B. Schumacher and J. Travis. Human Sperm Acrosomal Proteinase: Antibody Inhibition and Immunologic Dissimilarity to Human Pancreatic Trypsin. Fertil. Steril. 24, 479-484 (1973).

30. J. Travis and R. Pannell. Selective Removal of Albumin from Plasma by Affinity Chromatography. Clin. Chim. Acta 49, 49-52 (1973).

31. D. A. Johnson, R. N. Pannel and J. Travis. The Molecular Stoichiometry of Trypsin Inhibition by Human Alpha-1-Proteinase Inhibitor. Biochem. Biophys. Res. Comm. 57, 584-589 (1974).

32. J. Travis and R. Pannell. A Method for Preparing Albumin-Free Plasma. Behring Institute Mitteilungen 54, 30-32 (1974).

33. J. Travis and R. Pannell. Properties of Human Alpha-1-Anti-trypsin: Purification, Properties and Interaction with Human Pancreatic Trypsin. Bayer Symposium V, "Proteinase Inhibitors" 31-39 (1974).

34. J. Bieth, M. Aubry and J. Travis. The Interaction of Human Cationic Trypsin and Chymotrypsin II with Human Serum Inhibitors. Bayer Symposium V, "Proteinase Inhibitors" 53-62 (1974).

35. P. A. Mallory and J. Travis. Properties and Inhibition Spectrum of a New Pancreatic Protease. Bayer Symposium V, "Proteinase Inhibitors" 250-251 (1974).

36. R. Pannell, D. Johnson and J. Travis. Isolation and Properties of Human Plasma-α-1 Proteinase Inhibitor. Biochemistry 13, 5439-5445 (1974).

37. P. A. Mallory and J. Travis. Human Pancreatic Enzymes: Purification and Characterization of a Non-elastolytic Enzyme, Protease E, Resembling Elastase. Biochemistry 14, 0000-0000 (1975).

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R: REDACTED MATERIAL

CURRICULUM VITAE

NAME: Nancy Rosalie Matheson

REDACTED

RANK:

EDUCATION:

Year	Degree	Institution
1961	B.S.	University of Massachusetts
1964	M.A.	University of Massachusetts
1973	Ph.D.	University of Georgia

TITLE OF MASTER'S THESIS: "The Effects of Metabolic Inhibitors on Osmoregulation by Nauplii of Artemia salina at various salinities."

TITLE OF DOCTORAL DISSERTATION: "The Regulation of Fructose 1,6-diphosphatase from Swine Kidney."

EXPERIENCE:

Research Associate - University of Georgia, 1973-present, under Dr. James Travis.

Research Associate - University of Georgia, 1973-73, under Dr. John Lee.

Research Assistant - Ohio State University, 1967-68, under Dr. Joseph Mendicino.

Research Assistant - Columbia University, 1965-67, under Dr. F. C. G. Hoskin.

Research Assistant - University of Massachusetts, 1964-65, under Dr. J. L. Roberts.

PUBLICATIONS:

1. N. Kratowich and J. Mendicino. Role of enzyme-enzyme interactions in the regulation of gluconeogenesis. Effect of fatty acids, tricarboxylic acid cycle intermediates, and dinitrophenol on the rate of inactivation of D-fructose 1,6-diphosphatase by kidney mitochondria. J. Biol. Chem. 245, 2483-2492 (1970).

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2. J. Mendicino, N. Kratowich, and R. M. Oliver. Properties and subunit structure of D-fructose 1,6-diphosphatase. J. Biol. Chem. 247, 6643-6650 (1972).

3. H. Abou-Issa, N. Kratowich, and J. Mendicino. Properties of soluble and bound protein kinases isolated from swine kidney. Eur. J. Biochem. 42, 461-473 (1974).

4. J. Mendicino, H. Abou-Issa, R. Medicus, and N. Kratowich. Fructose 1,6-diphosphatase from swine kidney. Methods in Enzymology, Carbohydrate Metabolism, 1974 (In Press).

5. N. Kratowich and J. Mendicino. Role of enzyme interactions in the regulation of gluconeogenesis. Modification of the binding properties and activity of fructose 1,6-diphosphatase by adenosine monophosphate, adenosine triphosphate, and fructose 1,6-diphosphate. J. Biol. Chem. 249, 5485-5494 (1974).

6. I. B. C. Matheson, R. D. Ethridge, N. Kratowich, and J. Lee. The quenching of singlet oxygen by amino acids, and proteins. Photochem. and Photobiol., 1975 (In Press).

7. N. Kratowich and J. Mendicino. Effect of fatty acids, tricarboxylic acid cycle intermediates, and dinitrophenol on the rate of inactivation of D-fructose 1,6-diphosphatase. Abstracts of the Am. Chem. Soc. Meeting, Richmond, Virginia, November 5-8, 3 (1969).

8. N. Kratowich, J. Garner, and J. Mendicino. Regulation of D-fructose 1,6-diphosphatase activity by the oxidation of fatty acids and tricarboxylic acid cycle intermediates in kidney mitochondria. Fed. Proc. 29, 896 (1970).

9. N. Kratowich, H. Abou-Issa, and J. Mendicino. Regulation of glycogen synthetase activity in kidney by subcellular particulate and soluble protein kinases. Abstracts of the 162nd Am. Chem. Soc. Meeting, Washington, D.C., September 13-17, 251 (1971).

10. H. Abou-Issa, N. Kratowich, and J. Mendicino. Inactivation of glycogen synthetase by kidney cortex mitochondria. Abstracts of the 162nd Am. Chem. Soc. Meeting, Washington, D.C., September 13-17, 252 (1971).

11. N. Kratowich and J. Mendicino. Specific elution of fructose 1,6-diphosphatase by adenosine monophosphate and adenosine triphosphate. Abstracts of the 163rd Am Chem. Soc. Meeting, New York, N. Y., August 25-31, 207 (1972).

12. H. Abou-Issa, N. Kratowich, and J. Mendicino. Inactivation of kidney glycogen synthetase by soluble and particulate protein kinases. Fed. Proc. 31, 460 (1972).

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R: REDACTED MATERIAL

CURRICULUM VITAE

NAME: David Andrew Johnson

REDACTED

RANK: NIH Postdoctoral Fellow

EDUCATION:

Year	Degree	Institution
1967	B.S.	Memphis State University
1973	Ph.D.	Memphis State University

TITLE OF DOCTORAL DESSERTATION: "Purification and Properties of a Proteolytic Enzyme from Escherichia coli."

EXPERIENCE:

1973-present. Research Associate, University of Georgia

Summers, 1972-73. Instructor of Biochemistry for senior undergraduates and biology graduate students, Memphis State University.

1967-1973. Chemistry Department graduate teaching assistant, Memphis State University.

Summers, 1967-68. Physical Science Technician, Antigen Preparation Lab, TB Branch, National Center for Disease, Atlanta, Georgia.

PUBLICATIONS:

D. A. Johnson and William H. Spell. Purification and Properties of a Protease from Escherichia coli. Fed. Proc. 32, 1593 (1973).

J. Travis, D. A. Johnson, and R. Pannell. Properties of Human Alpha-1-Antitrypsin. Bayer Symposium V, "Proteinase Inhibitors" (1974).

D. Johnson, R. Pannell, and J. Travis. The Molecular Stoichiometry of Trypsin Inhibition by Human Alpha-1-Proteinase Inhibitor. Biochem. Biophys. Res. Comm. 57, 584-589 (1974).

J. Travis, R. Pannell, and D. Johnson. Interaction of Human α-1-Proteinase Inhibitor with Trypsin. Fed. Proc. 33, 1563 (1974).

10035463 99

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D. Johnson, J. Mendicino, M. Cormier, and J. Travis. Cyanukic Chloride: A New Coupling Reagent for Affinity Chromatography. Fed. Proc. 33, 1502 (1974).

R. Pannell, D. Johnson, and J. Travis. Purification and Properties of Human Alpha-1-Proteinase Inhibitor. Biochemistry

D. Johnson and J. Travis. Human Pancreatic Enzymes: Single Step Purification of Cationic Trypsin and Chymotrypsin I on Sepharose-Trasylol. Biochim. Biophys. Acta (Submitted).

D. Johnson and J. Travis. Mechanism and Structure of Alpha-1-Proteinase Inhibitor. XXIIIrd Colloquium, "Protides of the Biological Fluids," Brugge Belgium.

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CTR Grant No. 862R2

James Travis, Ph.D.
Department of Biochemistry
University of Georgia
Athens, Georgia 30602

Final Progress Report (July 1/72 - Dec. 31/74)

Biochemistry of Chronic Obstructive Lung Disease

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PROGRESS REPORT –

This report includes the results obtained from July 1, 1972 through Dec. 31, 1974 or about two and one-half years of the three year project which terminates June 30, 1975.

1. SUMMARY

The overall objectives of the original project were to determine whether the absence of α-1-antitrypsin (heretofore referred to as α-1-proteinase inhibitor or α-1-PI) resulted in abnormal degradation of lung alveoli by uncontrolled proteolysis due to elastase-like enzymes secreted from granulocytes or from the pancreas. The destruction of the alveoli, it was assumed, ultimately resulted in the development of emphysema.

The specific aims were to a) isolate and characterize human pancreatic elastase, b) isolate and characterize elastolytic enzymes from human purulent sputum and human granulocytes and compare each with that from the pancreas, c) to isolate human -1-PI from the two most phenotypically different types of individuals classified as MM and ZZ and study their comparative properties and their ability to interact with pancreatic and leukocytic proteases.

We have accomplished virtually all of the original goals of this project with the exception that we have chosen not to work with purulent sputum since it is far too complicated a mixture of bacterial and human proteins to make any definite conclusions about the sources and importance of enzymes found therein. The leukocytic elastase(s) have been purified, however, and a comparison with a human pancreatic enzyme is being made. The pancreatic enzyme, however, is not a true elastase.

Two forms of α-1-PI, from MM and ZZ plasma, have been isolated and preliminary structural studies indicate major differences in the carbohydrate side chain, although not in sialic acid content. Evidently, this deficiency, which results in the deposition of the abnormal protein (Type ZZ) in the liver, is due to an inborn error in carbohydrate side chain synthesis. Studies on the interaction of the normal inhibitor with several proteases, including leukocytic elastase, indicate that the molecule can bind two moles of enzyme per mole of inhibitor. This would suggest that the real concentration of α-1-PI in plasma is only about one-half of that currently accepted in the literature. The mechanism by which the interaction occurs is not yet known but studies have been initiated in this direction and a working hypothesis is presented below. (Section IIa)

Finally, in order to purify many of the proteins required for several of our studies, new, perhaps revolutionary, techniques for albumin removal from plasma, albumin isolation, protease purification, and general affinity chromatography have been developed. Details on all of the above information are given in subsequent sections.

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2. DETAILED PROGRESS REPORT

a) PURIFICATION OF α-1-PI AND STUDY OF ITS MECHANISM OF ACTION

The initial attempts to purify this protein by affinity chromatography (1) resulted, unfortunately, in the isolation of a pure but inactive protein. Since the major contaminant in the purification was due to albumin, we developed an affinity column containing the conjugate known as blue dextran. This material has a strong affinity for albumin and virtually no affinity for other plasma proteins. As a result we were able to prepare albumin-depleted plasma (2,3) (< 2% albumin left) which we were able to utilize for the subsequent purification of the inhibitor. This was performed by salt fractionation and two ion-exchange chromatography steps (4,5). The resulting material (molecular weight 52,000) had a capacity to bind two moles of trypsin, chymotrypsin, or elastase per mole of inhibitor (6).

Although some studies on the mechanism by which this inhibitor interacted with proteases have been carried out (7), the experiments are far from complete. Our present results indicate that with trypsin (molecular weight 25,000) a simple 1:1 covalently bonded complex of molecular weight 78,000 is first formed. This complex is then acted upon by a second molecule of trypsin to release a polypeptide chain of molecular weight 18,000, which immediately reacts with the trypsin molecule to give a new complex of molecular weight 43,000 (8,9). Such a mechanism involving limited proteolysis is not unusual and occurs in zymogen activation, blood clotting, and complement activations.

The type ZZ inhibitor has been isolated by the same procedure as that for type MM. Preliminary experiments indicate no differences in the amino acid composition of the two proteins but a significant change in carbohydrate composition. This portion of the α-1-PI molecule, which represents 12% of the molecular weight, has substantially less hexosamine content in the type ZZ protein (10). Thus, the apparent reason for α-1-PI (type ZZ) accumulation in liver is due to aberrant side chain synthesis due, presumably, to a defect in a glycosyl transferase in the liver Golgi apparatus where carbohydrate side chain synthesis is known to occur.

b) PURIFICATION OF GRANULOCYTIC ELASTASE

In order to determine if, indeed, elastase enzymes were involved in lung damage, it was necessary to purify this enzyme from both human granulocytes and pancreas.

The isolation of granulocytic elastase was immediately complicated by DNA and RNA binding which tended to precipitate the enzyme. However, by isolating granulocytes using dextran sedimentation and lysing the cells with heparin, we were able to obtain intact granules virtually free of DNA or RNA. Granule lysis in high salt resulted in a very active preparation of crude granulocytic elastase.

The purification of the enzyme(s) has been accomplished by taking into account the inhibitory properties of Trasylol (Kunitz bovine pancreatic trypsin inhibitor) towards granulocytic elastase activity. By preparing an affinity column of Sepharose-trasylol we were able to absorb all of the elastase activity and subsequently desorb by changing pH (from 6.5 to 4.0). This resulted in the isolation of four isoenzymes, all of which had elastolytic activity. A twenty-fold

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increase in specific activity occurred in this step. Subsequent ion-exchange chromatography on CM-cellulose resolved the major form of elastase (representing about 70% of the total activity) from the other three forms (11). We believe that proteolysis by other enzymes in granulocytes has caused the formation of the multiple enzymes. We are currently using only the major form for comparative studies which, to date, indicate no similarities between this enzyme and that from pancreas, described below.

The granulocyte elastase (MW 26,000) is rapidly inactivated by α-1-PI and the product has a molecular weight greater than 90,000 indicating direct formation of 2:1 complex. Further studies are in progress as described in the specific aims section of this proposal.

c) ISOLATION AND PROPERTIES OF PANCREATIC PROTEASES

In order to determine whether human pancreatic elastase might play some role in lung digestion or at least be related to the granulocytic enzyme in such a way as to make it useful in model systems, attempts were made to purify the protein. The results were certainly useful but also somewhat unusual. A protein with very similar properties to pancreatic elastase was purified (12,13). This protein, however, could only hydrolyze low molecular weight synthetic substrates as well as denatured proteins. It could not digest elastin. The reasoning behind the results obtained was that the human enzyme was too acidic in nature to bind to elastin despite the fact that it hydrolyzed similar peptide bonds in other proteins. The enzyme was found to be very dissimilar to the granulocytic elastase. However, it is probably very useful in the digestion of hydrophobic proteins in the intestine. A second protease with weak elastolytic activity is present in human pancreas but it is present in insufficient quantities to make isolation feasible.

During the isolation of the new enzyme which we called "protease E", a second form of human trypsin was isolated (14). This anionic protein was similar to cationic trypsin isolated in this laboratory several years ago. However, anionic trypsin is exceedingly unstable and autolyzes rapidly at alkaline pH. At acid pH it is irreversibly denatured. It is doubtful if this enzyme plays an important role in digestion.

d) REMOVAL OF ALBUMIN FROM PLASMA

As stated in section 2a, an important technique used in the purification of α-1-PI involved albumin binding on the insoluble support, sepharose-blue dextran. We have further investigated this phenomenon with very encouraging results. By using directly the chromophore of blue dextran, known as Cibacron Blue F-3GA, we have been able to prepare insoluble supports of this dye attached to sepharose, which can bind up to 30 mg of albumin per ml of packed gel derivative. Thus, on columns 5 x 100 cm we have been able to retain nearly all of the albumin in 500 ml of plasma without saturating the sepharose-dye derivative. A small quantity of albumin, presumably withits dye-binding site blocked because of the attachment of some other metabolite, always passes through the column. This albumin depleted plasma can be utilized for the purification of many plasma proteins usually contaminated by albumin. Other fluids containing albumin, such as cerebrospinal fluid, urine, saliva, tears, etc., could presumably be freed of this protein by

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adsorption on sepharose-Cibacron Blue F-3GA and the fluid then utilized for biochemical or physiological studies. We have used other dye derivatives similar to Cibacron Blue F-3GA with identical results.

The albumin can be isolated from these columns by addition of desorbents including K^+, Ca^{++}, Mg^{++}, octanoate, or SCN^-. The latter has proven extremely useful. The column can be re-equilibrated and re-used indefinitely (15). The albumin appears to be pure in form by electrophoresis and has been utilized by Dr. A. Humphries, Medical College of Georgia for stabilizing kidneys prior to transplantation. He has found it superior to any commercial preparations of albumin in that he has been able to keep dog kidneys viable for at least fourteen days by perfusing with our albumin.

e) INHIBITION STUDIES ON PANCREATIC PROTEASES

Because we have now purified six human pancreatic proteases to date in our laboratory, it was felt that a complete study of the inhibition spectra of naturally occurring inhibitors towards these enzymes might prove useful. The results have been submitted for publication (16).

In summary, it was found that human pancreatic proteases are, in general, poorly inactivated by naturally occurring inhibitors, including soybean trypsin inhibitor, Bowman Birk inhibitor, lima bean inhibitor, chicken ovomucoid, Kunitz Pancreatic Trypsin inhibitor, and Kazal Secretory Trypsin inhibitor. Only α-1-PI was a strong inhibitor of all human pancreatic proteases. On the other hand, protease E, the elastase-like protease, was very poorly inhibited by all inhibitors except α-1-PI.

From the above results we were able to determine that Trasylol (Kunitz Pancreatic Trypsin Inhibitor), attached to sepharose by cyanogen bromide activation, should be able to bind human cationic trypsin and one form of chymotrypsin. Acidification of the column would then result in release of the enzymes. By taking advantage of this fact we were able to design a single-step procedure for the isolation of human cationic trypsin and one form of human chymotrypsin, from activated pancreatic extracts, as pure entities separated from each other (17). This procedure completely superceded earlier methods developed in our laboratory.

f) INTERACTION OF α-1-PI WITH HUMAN THROMBIN

In order to determine what other roles α-1-PI might have in regulating proteolysis studies have been initiated on both the clotting and fibrinolytic system.

Although α-1-PI does not instantaneously inhibit thrombin, clotting times are considerably lengthened as incubation of the inhibitor with enzyme is increased. In fact, the slow formation of a complex can be established by SDS gel electrophoresis after 30 min. incubation. These studies are still in progress and are hindered by the necessity to work with highly purified human thrombin. Further studies with this enzyme and with human plasmin are planned and are described in the Specific Aims and Methods of Procedures section of this proposal.

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g) NEW TECHNIQUES IN AFFINITY CHROMATOGRAPHY

As a consequence of our work with the Cibacron dyes as affinity chromatography ligands we became interested in the backbone compound from which they are made. This molecule, cyanuric chloride, is highly reactive and can interact with NH_2, OH, and SH groups. Since it has three reactive chloride groups it can be attached to sepharose through one group, and to ligands through the other two chlorides since all three react at different temperatures. Thus, we have now made a variety of sepharose conjugates, heretofore unobtainable by classical cyanogen bromide activation, which whould prove useful in affinity chromatography for the purification of a number of enzymes. The derivatives we have made include sepharose-trasylol which works as well as that described above (section 2e, Ref. 17), sepharose-pectin, which is useful for amylase isolation, sepharose-riboflavin which is being utilized for the isolation of flavoenzymes by others in our department and a number of sepharose dye derivatives which bind the bioluminescent enzymes called luciferases. It should be noted that the attachment of carbohydrates to sepharose in a direct manner has never been described before.

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REFERENCES

1. R. Pannell and J. Travis (1973), *Fed. Proc. 32,* 504 Abs.

2. J. Travis and R. Pannell (1973), *Clin. Chim. Acta 49,* 49.

3. J. Travis and R. Pannell (1974), *Behring Inst. Mitt. 54,* 30.

4. R. Pannell, D. Johnson, and J. Travis (1974), *Biochem. 13,* 5439.

5. J. Travis, D. Johnson, and R. Pannell (1974), *Bayer Symposium V "Proteinase Inhibitors," 31,* Springer-Verlag (1974).

6. D. Johnson, R. Pannell, and J. Travis (1974), *Biochem. Biophys. Res. Commun. 57,* 584.

7. J. Bieth, M. Aubry, and J. Travis (1974), *Bayer Symposium V "Proteinase Inhibitors," 53,* Springer-Verlag (1974).

8. J. Travis, R. Pannell, and D. Johnson (1973), *Fed. Proc. 33,* 1563 Abs.

9. D. Johnson and J. Travis (1975), Abs. XXIIIRO Colloquium - *Protides of the Biological Fluids.*

10. J. Travis and R. Pannell (1975), Abs. XXIIIRO Colloquium - *Protides of the Biological Fluids.*

11. R. Baugh and J. Travis (1975), *Fed. Proc.* (Abs., In Press).

12. P. A. Mallory and J. Travis (1974), *Bayer Symposium V "Proteinase Inhibitors," 250,* Springer-Verlag (1974).

13. P. A. Mallory and J. Travis (1975), *Biochem.* (In Press).

14. P. A. Mallory and J. Travis (1973), *Biochem. 12,* 2847.

15. J. Travis, R. Pannell, N. Matheson, and D. Johnson (1974), *Nature* (Submitted for publication).

16. P. A. Mallory and J. Travis (1975), *Amer. J. Clin. Nutrition* (Submitted for publication).

17. D. Johnson and J. Travis (1975), *Biochim. Biophys, Acta* (Submitted for publication).

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THE UNIVERSITY OF GEORGIA

Department of Biochemistry
BOYD GRADUATE STUDIES RESEARCH CENTER
ATHENS, GEORGIA 30602

TELEPHONE 404 542-1334

February 6, 1975

Dr. V. Lisanti
The Council for Tobacco Research, U.S.A.
110 East 59th Street
New York, N. Y. 10022

Dear Dr. Lisanti:

Pursuant to our telephone conversation of a few days ago, I am writing to inform you of some experiments we are attempting in order to rapidly phenotype individuals as potential candidates for the development of emphysema. We are assuming that type ZZ and type MM α-1 proteinase inhibitor (α-1 antitrypsin) phenotypes would be most prone to the disease. Our experimental approach is multi-folded:

a) We have purified type ZZ α-1-PI and we are making antibodies to this protein as well as to type MM on which we have already reported. We hope that the Z protein may have specific antigenic sites not available on the M protein. Hence, it may be possible to isolate directly or by immunoabsorption a monospecific antigen to the Z protein which could be utilized for mass screening of the population by simple immunoprecipitation techniques.

b) We are developing electrophoretic techniques for the direct identification of MZ and ZZ plasma protein patterns. We have several approaches in mind, although we have, at the moment, initiated studies on only a few.

1. We knew that acrylamide slab gel electrophoresis at pH 5.0 gives some separation of the ZZ and MZ protein patterns which can be distinguished. Albumin, however, does cause some interference.

2. We can remove the albumin by the techniques we have developed and perhaps increase the separation to the sensitivity we desire. It is this approach we are favoring at the moment.

3. We have discussed the possibility of using "oblique thinking" and adding trypsin to plasma to form α-1-PI-trypsin complexes which will migrate in distinctively different positions. It may be that due to the charge differences in the various α-1-PI phenotypes we may be able to see interpretable patterns for the various complexes formed. We have not yet started this work.

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We feel that if our techniques work as well as preliminary results indicate, a properly equipped laboratory should be able to analyze at least 48 samples in an eight hour day. The more electrophoresis equipment available, however, the larger the number of samples which could be analyzed.

With the immunodiffusion technique, of course, the sample number is increased immeasurably.

I hope this update will be useful to you and to the Council for Tobacco Research-U.S.A.

Sincerely yours,

James Travis
Associate Professor

JT:pdc

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PHARMACOLOGY

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#1015 – INGENITO

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

January 9, 1975

Grant application No. 1015

PHARMACOLOGY

To: The committee comprising Drs. Bing, Sommers and Wyatt

Subject: Alphonse J. Ingenito, Ph.D., The Albany Medical College of Union University, Albany, New York
New application No. 1015
"Actions of Carbon Monoxide and Tobacco Smoke on Retinal Metabolism and Function".

History

An informal inquiry was handled as Case No. 299. The Executive Committee voted to encourage formal application.

Request

Application No. 1015 requests $29,910 for the first year of a two-year project: This amount includes $4,320 for permanent equipment.

Documents submitted

1. Application dated December 30, 1974 (23 pages)
2. CV of Dr. Ingenito (4 pages)
3. Two reprints and five abstracts

D.S.

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1015

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 12/30/74

JAN 20 1975

1. Principal Investigator (give title and degrees):

Alphonse J. Ingenito, Ph.D.
Associate Professor of Pharmacology

2. Institution & address:

The Albany Medical College of Union University
Albany, New York 12208

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Actions of Carbon Monoxide and Tobacco Smoke on Retinal Metabolism and Function

5. Proposed starting date: July 1, 1975.

6. Estimated time to complete: Two years.

7. Brief description of specific research aims:

The project to be described will assess, in the acute experimental situation, the effects of tobacco smoke and carbon monoxide (CO) at concentrations comparable to those occurring in tobacco smoke, on retinal metabolism and function. The main aim will be to determine whether the deficits in visual function reported to occur following inhalation of small concentrations of CO or heavy tobacco smoking, might have their origin in the retina, specifically on certain CO-sensitive biochemical functions of the pigment epithelium of retina (to be described below). Using both in vitro and in vivo physiological and biochemical systems in bovine, porcine, feline and rabbit eyes, an attempt will be made to correlate any pharmacological effects observed on retinal function with those observed on biochemical processes. An attempt will also be made to relate the data to the possibility of deficits in visual function caused by heavy smoking and exposure to environmental levels of CO.

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8. Brief statement of working hypothesis:

The hypotheses to be tested are: (1) that tobacco smoke and CO cause deficits in the function of the visual system by direct actions on the retina, rather than elsewhere in the neural visual pathways and brain; (2) that these effects are not directly due to carboxyhemoglobin (HbCO) formation with loss of blood O_2 carrying function but are due instead to biochemical effects of CO on the retina itself; (3) that a major biochemical effect of tobacco smoke and CO on the retina is on a cytochrome p-450 microsomal electron-transporting oxidase system in the pigment epithelium which might be involved in retinol-retinaldehyde interconversions and (4) that these effects occur at tissue concentrations of CO theoretically attainable in the smoking human.

9. Details of experimental design and procedures (append extra pages as necessary)

The carbon monoxide (CO) content of tobacco smoke has been estimated at between 2 and 6%, depending upon the type of tobacco product and the manner of smoking (Wynder and Hoffmann 1967). Such concentrations, when inhaled by heavy smokers, could conceivably result in blood carboxyhemoglobin (HbCO) levels as high as 10% (van Proosdij 1960). A variety of biological effects of such concentrations, or even lower, have been reported and many of these are said to be detrimental to health (see, eg studies cited by Larson et al., 1961, 1968, 1971; Goldsmith and Landaw, 1968 and Ayres et al., 1969). One biological system especially sensitive to CO is the visual system, and effects on visual acuity and thresholds (Halperin, McFarland et al., 1959 and later studies by the McFarland group; Beard and Grandstaff, 1970) are observed at HbCO levels well within the smoking range, i.e. 5% or even less. The subject of "tobacco amblyopia" is, after a stormy history beginning in the late 1800's, still a debated issue. Although some authorities do not consider this to be a true tobaccogenic disease and believe it to be due, rather, to nutritional deficiencies sometimes associated with heavy tobacco usage, others still maintain that visual defects are a direct consequence of heavy smoking (studies as cited by Grant, 1974; Larson et al., 1961, 1968, 1971 and Shapero 1971). According to some accounts, tobacco amblyopia has the peculiarity of being associated with heavy cigar and pipe smoking and rarely with cigarette smoking (Rucker and Kearns, 1961 as cited by Larson and Sylvette, 1968, pg. 382). Since cigar and pipe smoke are said to contain more CO than cigarette smoke due to incomplete combustion (van Proosdij, 1960, Wynder and Hoffmann 1967), this may account for the latter finding.

The disturbances of visual function often seen following severe CO intoxication are usually attributed to effects on the visual areas of the brain (Grant, 1974, LaPresle and Fardeau, 1967 and other studies in the same volume) but the site(s) of action of CO in the visual system following the milder exposures to CO as with smoking and environmental concentrations, although sometimes ascribed to the brain (Beard and Grandstaff, 1970) are in fact unknown (Halperin et al., 1959). Since the retina is sensitive to toxic agents in a very selective fashion (Potts, 1965, Grant, 1974) it seemed to us to be a likely candidate for some selective biochemical action of CO. The ability of CO to bind to heme pigments such as hemoglobin and the cytochromes is

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9. Details of experimental design and procedures - Continued

well known, but it is difficult to conceive how a blood HbCO of eg. 5% in a smoker, might result in a visual decrement due to the slight diminution of blood oxygen carrying capacity, and slight shift of the HbO_2 dissociation curve to the left, which this low level might cause. Lilienthal (1950) has pointed out that it is at least theoretically possible for CO to exert some of its effects via binding to tissue cytochromes, but this is generally dismissed as improbable due to insufficient blood concentrations of dissolved CO even at severe levels of CO intoxication (see, eg, discussion by LaPresle and Fardeau, 1967). This latter view, however, neglects the possibility for active transport mechanisms for CO into tissues as has in fact been shown to occur in lung and placenta (Burns and Gurtner, 1968) mediated by cytochrome p-450 which apparantly functions as a carrier for both CO and O_2 from blood to tissues. Also, in our own laboratory we (Ingenito et al, 1974) have demonstrated a long-lasting negative ionotropic action of CO on the perfused isovolumic rabbit heart with a hemoglobin free perfusate. This study established that CO can be carried into tissues from a dissolved state and can be bound to some myocardial tissue component, possibly myoglobin or the cytochromes. That a CO-cytochrome complex might be responsible for the effects of CO in the visual system is given further impetus from the statement of Halperin et al.,(1959) "The results described (for CO and the visual system)... appear to point definitely to the presence, somewhere in the visual and/or nervous systems of some hitherto unrecognized substance with hematin-like properties and of marked physiological importance in vision." The presence and importance of such a substance was recently demonstrated by Shichi (1969) who found a cytochrome p-450 in the microsomal frac-

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9. Details of experimental design and procedures - Continued

tion of pigment epithelial cells in bovine retina. Shichi showed that this CO-sensitive cytochrome functioned as part of an electron-transport system which catalyzed the oxidation of NADPH and NADH and he suggested that it might function in the retina to effect retinol-retinaldehyde interconversion, an important component of the rhodopsin cycle in visual cells. (Wald 1968.) The pigment eptihelium is considered to be of prime importance in mediating the exchange of blood borne nutrients and gases between the choroidal circulation and the rods and cones and to carry out essential metabolic and supporting functions for the visual cells. (Bernstein 1960, Porter and Yamada 1960, Moyer 1969). Its destruction by natural causes (retinitis pigmentosa) or by chemical agents (Potts, 1965) can lead to blindness.

Experimental Design and Procedures

The protocol to be described will attempt to correlate biochemical effects in an in-vitro system with possible pharmacological actions of CO and tobacco smoke on in-vivo preparations. The key hypotheses to be tested are: (1) that an effect of CO and/or tobacco smoke on visual function can be defined at the level of the retina itself using acute, in-vivo and in-vitro preparations and (2) that an effect of CO and/or tobacco smoke can be described on a cytochrome-containing enzyme system in pigment epithelium which converts retinol to retinaldehyde.

I. Electrophysiological Studies

To establish actions on the retina for CO and/or tobacco smoke, three preparations are contemplated:

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9. Details of experimental design and procedures - Continued

1. Electroretinogram of the Anesthesized Cat:

We plan to monitor the electroretinogram (ERG) of the anesthetized cat (pentobarbitol or chloralose, depending upon which causes less interference with ERG recordings) made to inhale various concentrations of CO in oxygen or air, so as to allow arterial blood HbCO levels to rise to "smoking levels" (between 5 and 15%). The ERG will be evoked by photic stimulation (Grass photostimulator). The results will be compared with exposures to similar degrees of hypoxia induced with nitrogen so as to establish whether the effects are secondary to hypoxia or are due instead to some other action of CO. Cats will also be made to inhale 25 ml puffs of tobacco smoke from University of Kentucky research cigarettes and the ERG monitored. The ERG is considered to be a good indicator of retinal function and its various components have been intensely investigated as to their origins in discrete layers of the retina (Davson 1972, Brown, 1968, Brown and Wiesel, 1959, Jacobson 1961, Crescitelli 1960, Noell 1954 and many others). The c-wave of the ERG has particular importance for the proposed study since its origin is said to be the pigment epithelium and it is depressed by toxic agents such as sodium azide, and sodium iodate which selectively destroy pigment epithelium. (Noell, 1954; Potts, 1965) The existing literature (op cit above and others) on anesthetic and other drug effects on the ERG will serve as a guide in the interpretation of the ERG. The recording of the ERG is a relatively simple and non-invasive technique which preserves the functional status of retina and pigment epithelium. We will most likely be able to use our existing equipment for this with the exception of some corneal and other electrodes and a

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9. Details of experimental design and procedures - Continued

photostimulator which will need to be purchased. A Tektronix Dual Beam Oscilloscope with Polaroid Camera or a Beckman Dynograph will be used to record the electrical potentials involved.

2. Isolated Perfused Cat Eye (Hemoglobin-Free Perfusate):

The above intact cat preparation, although important to establish the effects of CO and tobacco smoke on the relatively intact eye, presents some problems in interpretation because the effects of HbCO, however slight at the low levels employed, complicate interpretations somewhat. To establish the possibility of direct effects of CO on the retina, and to exclude the possibility of HbCO-induced hypoxia, it will be necessary to assess retinal function in a hemoglobin-free system. The isolated perfused cat eye preparation of Gouras and Hoff (1970) offers a unique approach since the ERG may be recorded in the intact eye after removal from the animal and perfusion in a bath with a hemoglobin-free physiological salt solution under controlled conditions. The optics of this system are rather complex and will need simplification in our approach to its construction. The perfusion system (via the ophthalmociliary artery) is rather simple and should be adaptable to our existing perfusion equipment with the exception of the perfusion chamber which we will need to have constructed. The preparation is said to maintain normal ERG configurations for many hours; well within the time period necessary for us to demonstrate effects of CO and tobacco smoke. The perfusing solution will be bubbled with various concentrations of CO (or tobacco smoke) and the effects again compared to equivalent degrees of nitrogen hypoxia.

3. Isolated Retina-Optic Nerve Preparation (Rabbit Eye):

The previously described perfused cat eye preparation of Gouras and Hoff will provide some insight as to CO effects on the retina in

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9. Details of experimental design and procedures - Continued

a hemoglobin free system, in which any effects in the pigment epithelium would be manifested in the overall results. It would be desirable to also utilize a preparation to monitor electrophysiological functions in the retina alone, i.e. without the pigment epithelium, so as to evaluate the contribution of the latter structure in the overall effect of CO. The isolated retina-optic nerve preparation of Ames and Gurian (1960) offers this advantage as well as the advantage of using another smaller, less expensive species in which retinal function has been well studied, in which repeated in vitro studies can be made, and in which effects on the optic nerve as well as the retina might be assessed. Although the retinal potentials recorded with this preparation bear some resemblance to the conventional ERG, they cannot be totally equated. However, Ames has shown this light-stimulated preparation to be stable over a number of hours, to be very sensitive to O_2 lack and glucose deprivation, and to be a useful preparation to study correlations between the metabolic integrity and the functional aspects of retina. (Ames, 1965).

II. Biochemical Studies

The biochemical studies on retina will center around two techniques:

1. Studies on suspensions of and microsomal preparations of bovine and/or porcine pigment epithelial cells.

The studies will be designed to determine whether carbon monoxide, tobacco smoke, or aqueous extracts of tobacco smoke can inhibit the enzymatic conversion of retinol to retinaldehyde by the cytochrome p-450 microsomal oxidase system. This will be done in two ways: In the first method, we will prepare suspensions of pigment epithelial cells

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9. Details of experimental design and procedures - Continued

as described by Glocklin and Potts (1962) and incubating these, in suitable buffers equilbrated with CO, smoke or N_2 mixtures or tobacco smoke, in the presence of added quantities of retinol, in order to study the conversion of retinol to retinaldehyde both in the medium and in the cells. Retinaldehyde will be determined by the colormetric method of Futterman and Saslow (1961) and retinol by the colormetric method of Futterman (1963). If inhibition is detected with CO or tobacco smoke, we will attempt to determine the lowest concentration of CO or aqueous tobacco smoke extracts necessary to cause a measurable inhibition of retinaldehyde to retinol interconversion. Shichi (1969) makes no mention of the requirements for the presence or absence of light in his microsomal assay system so this aspect will need to be investigated. It would appear, offhand, that the reaction would proceed best in the dark, as it does in the natural state.

If time allows, we will also make some comparative studies, in the dark, of the ability of bovineor porcine retinal visual cell outer segments prepared according to Futterman (1963) or McConnell (1965) to conduct the same reaction, since all authorities do not agree that retinol-retinaldehyde conversion takes place only in pigment epithelium (Bridges, 1970).

The above evaluations will be carried out either ina Warburg type apparatus (Gilson Respirometer) which we have on hand or, more simply in a Dubnoff metabolic shaker, which will need to be purchased, but which is quicker to use and will allow more determinations in the same run.

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In the second technique for studying pigment epithelium biochemistry,

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9. Details of experimental design and procedures - Continued

we plan some limited work using Shichi's (1969) method for preparing microsomal fractions of pigment epithelial cells, in which the enzyme preparation is obtained in more concentrated form, allowing a study of the associated reduction of NADH or NADPH, this being an integral part of the oxidation of retinol. Changes in the spectrum caused by conversion of oxidized to reduced pyridine nucleotides may be determined in the spectrophotometer. This technique will also allow us to examine the spectral characteristics of the cytochrome p-450 containing enzyme in suspension. If the p-450 fraction is involved in the inhibition there should be bands of spectral absorbtion detectable at 447 and 470 mμ (Shichi, 1969). With some precautions taken to maintain a CO-atmosphere during the transfer of incubation mixture to cuvette, we should be able to detect evidence of CO-hemeprotein complexes in our microsomal reaction mixtures. This should constitute prima facie evidence of CO-induced enzyme inhibition.

For determining the spectral curves we will need to obtain a strip-chart recorder for our Beckman DB spectrophotometer since none is presently on hand. Also, we will need to use a high speed ultracentrifuge for preparing the microsomal fractions, which is not available in the Department of Pharmacology, but we have received permission from the Chairman of the Biochemistry Department here to use their Beckman models L-1 and L-2 ultracentrifuges for this purpose.

The Albany area is presently lacking a bovine abbatoir facility that does a sufficient volume of daily slaughtering to provide us with the approximately 50 bovine eyes, necessary to provide enough pigment epithelia to conduct the projected studies, since this structure is

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9. Details of experimental design and procedures - Continued

but a tiny layer of the retina. However, the Tobin Packing Company, a large pork abbatoir in our area, has agreed to supply us with freshly removed porcine eyes for this purpose. We will therefore adapt all published methods for bovine eyes to studies on porcine eyes instead. We can find no reason at present why the porcine eye should not serve equally well. We will, however, attempt to obtain enough bovine material for some preliminary studies comparing the pigment epithelia of both species.

2. Studies on Incubated Rabbit Retina

The metabolism of rabbit retina has been intensively studied and much is known about its carbohydrate and energy metabolism (Graymore 1969). We have already done some preliminary studies on the effects of CO on glucose uptake and lactate production in the light adapted retina, but the data is too little to detect any pattern of effect, as yet. We plan to continue these studies, using either the Warburg Technique (Cohen and Noell, 1960) or by using the Dubnoff metabolic shaker (Kornblueth et al., 1953, Baeyens et al., 1971) concentrating on ATP and glucose utilization and on lactate and pyruvate production in the incubation media equilibrated with N_2 or CO mixtures or with tobacco smoke or aqueous tobacco smoke extracts. The object here will be to determine whether carbohydrate energy metabolism, in general, is affected by CO or tobacco smoke, so as to provide alternative sites of action for CO other than those we have postulated. It might, e.g., be equally plausible that CO could bind to some cytochrome in the electron-transport chain of retinal visual cell mitochondria and thus diminish ATP production there, eventually disrupting membrane processes and other aspects of energy metabolism as reflected by a decreased

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9. Details of experimental design and procedures - Continued

glucose utilization and depletion of ATP stores in the medium and tissue. Alternatively, an action on some other transporting mechanism or carrier for O_2, between the medium and the tissue, might force the retina to anaerobic glycolysis, increased glucose utilization and increased lactate production. Lactate production is said to be especially critical in the retina because the acidity which it produces in the extracellular space may be of importance in maintaining a high tissue pO_2 in the relatively avascular retina via a Bohr shift in the HbO_2 dissocation curve, (Baeyens, et al., 1971). Pyruvate production will be studied as well since lactate/pyruvate ratios are considered to be a good indicator of the oxidative energy state of the tissue. ATP, glucose, lactate and pyruvate levels in both retinal tissue and incubation media will be done using established methods already being used in this laboratory. Since light might influence these reactions in the isolated retina, it will be necessary to conduct the incubations in both light and dark and in retinae taken from both light and dark adapted rabbits.

Under optimal conditions, the studies outlined above should take a full two years. We expect that during the first year considerable progress would be made on the biochemical aspects, since this is work with which we are familiar and have existing equipment to work with. During the first year we will need to have built, to assemble, and to test the equipment needed for the physiological studies on retina, including that for electroretinography and optic nerve potentials in the isolated retina-optic nerve preparation and the associated baths and perfusion devices. By the second year of the project we should be

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9. Details of experimental design and procedures - Continued

ready to collect data on the physiological aspects. It should be mentioned in this context that Mr. Arnold Rabin, presently a student in our second year medical class, and who is associated with the Department of Ophthalmology at Albany Medical College and occupies laboratory space in the Pharmacology Department, is an expert on electroretinography, has published studies on this subject (Rabin and Bersen, 1974) and has indicated a willingness to help set-up and run the instrumentation for electroretinography in the intact cat and perfused cat eye.

We could easily conceive that the above project might lead us to additional studies on related aspects of the smoking and vision problem, possibly involving clinical ERG studies with smoking humans, more detailed studies on retinal biochemistry and an analysis of the relative susceptibilities of different areas of the visual pathways to CO, a study somewhat analogous to the elegant analysis of the effects of anoxia on the various areas in the visual pathways by Noell and Chinn (1950). Also in view of our recent findings (Ingenito et al., 1975) that the effects of combinations of CO and nicotine in the rabbit heart differed from the effects of either agent alone, some studies on combinations of CO with other tobacco smoke constituents on the visual system would seem to be of importance.

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A casual glance through Larson et al.'s Tobacco monographs will convince the reader that the main emphasis of the tobacco and health problem has been placed on the effects of tobacco smoking on the cardiovascular and respiratory systems with relatively little emphasis on other systems. Perhaps it is time for some studies on those systems having a demonstrated sensitivity to certain of the active components

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9. Details of experimental design and procedures - Continued

ready to collect data on the physiological aspects. It should be

of tobacco smoke. We submit that it is time to give the visual

system some detailed consideration. [illegible] associated with the

Department of Ophthalmology at Albany Medical College and occupies

laboratory space in the Pharmacology Department, is an expert on

electroretinography, has published studies on this subject (Rabin and

Bersen, 1974) and has indicated a willingness to help set-up and run

the instrumentation for electroretinography in the [illegible]

[illegible]

We could [illegible] the above project [illegible] to

[illegible] the smoking and vision problem,

[illegible]

[illegible] relative

susceptibility [illegible]

study somewhat analogous to the elegant analysis of the effects of

anoxia on [illegible]

(1950). Also in view of our recent findings (Ingenito et al., 1975)

[illegible] effects of combinations of CO and nicotine in the [illegible]

[illegible]

on combinations of CO with [illegible]

[illegible]

[illegible]

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REFERENCES

1. Ames, A. Studies of Morphology, Chemistry and Function in Isolated Retina, Pg. 22-29, Biochemistry of the Retina, Graymore, C. N., Ed., Academic Press, N.Y., 1965.

2. Ames, A. and Gurian, B.S. Measurement of Function in an In-Vitro Preparation of Mammalian Central Nervous System, J. Neurophysiol., 23: 676-691, 1960.

3. Ayres, S. M.; Mueller, H. S.; Gregory, J. J.; Giannelli, S. Jr.; and Penny, J. L. Systemic and Myocardial Hemodynamic Responses to Relatively Small Concentrations of Carboxyhemoglobin, Arch. Env. Health, 18: 699-709, 1969.

4. Baeyens, D. A.; Hoffert, J. R. and Fromm, P. O. Aerobic Glycolysis and Its Role in Maintenance of High O_2 Tensions in the Teleost Retina, Proc. Soc. Exp. Biol. Med., 137: 740-744, 1971.

5. Beard, R. R. and Grandstaff, N. Carbon Monoxide Exposure and Cerebral Function, Acad. Sci., 174: 385-395, Ann, N.Y., 1970.

6. Bernstein, M. H. Functional Architecture of the Retinal Epithelium Pg. 139-150, The Structure of the Eye, Smelser, G. K., Ed., Academic Press, N.Y., 1960.

7. Bridges, C.D.B. Biochemistry of Vison, Chapter 9, Biochemistry of the Eye, Graymore, C. N., Academic, N.Y., 1970.

8. Brown, K. T. The Electroretinogram: Its Components and Their Origins, Vision Res., 8: 633-677, 1968.

9. Brown, K. T. and Wiesel, T. N. Intraretinal Recording with Micropipette Electrodes in the Intact Cat Eye, J. Physiol., 149: 537-562, 1959.

10. Burns, B. and Gurtner, G. H. A Specific Carrier for Oxygen and Carbon Monoxide in the Lung and Placenta, Drug Metab. and Disposition, 1: 374-377, 1973.

11. Cohen, L. H. and Noell, W. K. Glucose Catabolism of Rabbit Retina Before and After Development of Visual Function, J. Neurochem., 5; 253-276, 1960.

12. Crescitelli, F. Physiology of Vision, Ann. Rev. Physiol., 22: 525-578, 1960.

13. Davson, H. Electrophysiology of the Retina, Part 4, The Physiology of the Eye, 3rd Ed., Academic Press, N.Y., 1972.

14. Futterman, S. Metabolism of the Retina, III the Role of Reduced Triphophopyridine Nucleotide in the Visual Cycle, J. Biol. Chem. 238: 1145-1150, 1963.

1003546426

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15. Futterman, S. and Saslow, L.D. The Estimation of Vitamin A Aldehyde with Thiobarbituric Acid, J. Biol. Chem., 236: 1652-1657, 1961.

16. Glocklin, V.C. and Potts, A.M. The Metabolism of Retinal Pigment Cell Epithelium, Invest. Ophthalmol., 1: 111, 1962.

17. Goldsmith, J.R. and Landaw, S.A. Carbon Monoxide and Human Health, Science, 162: 1352, 1968.

18. Gouras, P. and Hoff, M. Retinal Function in an Isolated, Perfused Mammalian Eye, J. Inves. Ophthalmol., 9: 388-399, 1970.

19. Grant, M.W. Toxicology of the Eye, 2nd Ed., C.C. Thomas, Springfield, Carbon Monoxide, Pg. 235-241; Tobacco Smoking, Pg 1018-1023, 1974.

20. Graymore, C.N. General Aspects of the Metabolism of the Retina, The Eye, Chapter 8, Vol. 1, Davson, H., Ed., Academic Press, N.Y., 1969.

21. Halperin, M.H.; McFarland, R.A.; Niven, J.I. and Roughton, F.J.W. The Time Course of the Effects of Carbon Monoxide on Visual Thresholds, J. Physiol., 146: 583-593, 1959.

22. Ingenito, A.J.; Fiedler, P.C. and Procita, L. Negative Inotropic Action of Carbon Monoxide on the Isolated Isovolumic Heart with a Hemoglobin Free Perfusate, Fed. Proc., 33: 503, 1974. (abstract)

23. Ingenito, A.J.; Ryon, J.J.; and Procita, L. Carbon Monoxide-Nicotine Interactions on the Isolated, Perfused, Isovolumic Rabbit Heart, Fed. Proc., abstract in the press, 1975.

24. Jacobson, J.H. Clinical Electroretinography, C.C. Thomas, Springfield, 1961.

25. Kornblueth, W.; Yardeni-Yaron, E. and Wertheimer, E. Glucose Utilization of the Retina, A.M.A. Arch. Ophthalmol., 50: 45-49, 1953.

26. LaPresle, J. and Fardeau, M. The Central Nervous System and Carbon Monoxide Poisoning, II Anatomical Study of Brain Lesions Following Intoxication with Carbon Monoxide, Prog. Brain Res., 24: 31-74, 1967.

27. Larson, P.S.; Haag, H.B. and Sylvette, H. Tobacco, Experimental and Clinical Studies, Williams and Wilkins, Baltimore, 1961, also Supplement 1, 1968, and Supplement 2, 1971, Larson, P.S. and Sylvette, H.

28. Lilienthal, J.L., Jr. Carbon Monoxide, Pharmacol. Rev., 2: 324-354, 1950.

29. McConnell, D.G. The Isolation of Retinal Outer Segment Fragments, J. Cell Biol., 27: 459-473, 1965.

30. Moyer, F.H. Development, Structure and Function of the Retinal Pigmented Epithelium, The Retina, Straatsma, B.R.; Hall, M.O.;

1003546427

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30. Allen, R.A. and Crescitelli, F., Eds., Univ. Calif. Press, Berkeley, 1969.

31. Noell, W.K. The Origin of the Electroretinogram, Amer. J. Ophthalmol., 38: 78-90, 1954.

32. Noell, W. and Chinn, H.I. Failure of the Visual Pathway During Anoxia, Amer. J. Physiol., 161: 573-590, 1950.

33. Porter, K.R. and Yamada, E. Studies on the Endoplasmic Reticulum, V. Its Form and Differentiation in Pigment Epithelial Cells of the Frog Retina, J. Biophys. Biochem. Cytol., 8: 181-205, 1960.

34. Potts, A.M. Selective Action of Chemical Agents on Individual Retinal Layers, Biochemistry of the Retina, Graymore, C.N., Ed., Pg. 155-161, Academic Press, N.Y., 1965.

35. Rabin, A.R. and Berson, E.L. A Full-Field System for Clinical Electroretinography, Arch. Ophthalmol., 92: 59-63, 1974.

36. Rucker, C.W. and Kearns, T.P. Tobacco Amblyopia, Amer. J. Ophthalmol., 51: 509-510, 1961.

37. Shapero, M. Amblyopia, Chilton, Philadelphia, 1971.

38. Shichi, H. Microsomal Electron Transfer System of Bovine Retinal Pigment Epithelium, Exptl. Eye Res., 8: 60-68, 1969.

39. Van Proosdij, C.J. Smoking: Its Influence on the Individual and Its Role in Social Medicine, Elsevier, Amsterdam, 1960.

40. Wald, G. Molecular Basis of Visual Excitation, Science, 162: 230-239, 1968.

41. Wynder, E.L. and Hoffmann, D. Tobacco and Tobacco Smoke, Academic, N.Y., 1967.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The principal investigator has two laboratories and an office presently assigned to him totaling about 650 sq. ft. of floor space with the option of an additional laboratory of about 150 sq. ft. for studies in a dark environment, should the grant be approved and funded. The laboratories are well equipped with routine glassware, chemicals, refrigerators, water, electricity and vacuum lines and hoods for venting noxious gases. Equipment on hand necessary for these studies include a Beckman DB Spectrophotometer, Lourdes and International refrigerated centrifuges and rotors, Gilson Differential Respirometer, Beckman Offner Type R Dynograph with appropriate couplers and preamplifers, Tectronix Dual-Beam Oscilloscope, Polaroid oscilloscope camera, Beckman Blood pH meter, Radiometer pH meter, Instrumentation Laboratories Model 182 CO-Oximeter, Radimeter blood-gas monitor and electrodes, various gas handling valves and mixing equipment, two Tamson constant - temperature water baths, various glass reservoirs and other items needed for extracorporeal perfusions, and two Barnstead copper water stills for distilled water. The College's accredited animal facilities occupy the entire basement of two buildings in the Medical College Complex and will provide expert care and handling of animals. There is also a computer facility available for statistical analyses and the Department of Pharmacology has a small Hewlett-Packard Computer programmed for routine statistical analyses.

11. Additional facilities required:

As stated above an ultracentrifuge providing forces of 100,000 x g is required for the studies on pigment epithelium microsomal fractions. The cost of such an instrument with appropriate rotors is approximately $12,000, a price we feel to be disproportionate to the relative necessity of it for the proposed study. We have obtained permission to use an ultracentrifuge for that aspect of the study from the Chairman of the Department of Biochemistry where such a centrifuge is located.

12. Biographical sketches of investigator(s) and other professional personnel (append):

A curriculum vitae is attached.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):

See attached curriculum vitae, two papers and five abstracts.

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
A. J. Ingenito, Ph.D.	50	$ 5,000
Technical		
To be recruited	100	$ 9,000
Sub-Total for A		$14,000
B. Consumable supplies (by major categories)		
Reagents, biochemicals, gases, etc.		$ 2,000
Glassware, routine		$ 1,000
Perfusion & chromatography apparatus, glassware		$ 1,000
Surgical instruments & supplies		$ 500
Sub-Total for B		$ 4,500
C. Other expenses (itemize)		
Animals, cats, and rabbits and animal care		$ 2,000
Bovine or porcine eyes from local abbatoir		750
Photographic & illustration work for manuscript preparation		400
Computer analyses of data		100
Travel to meetings		500
Sub-Total for C		$ 3,750
Running Total of A + B + C		$22,250
D. Permanent equipment (itemize)		
Glass distilled water apparatus		600
Grass Photostimulater		550
Dubnoff Metabolic Shaker		850
Misc. items of electronic & gas delivery equipment		750
Lucite chambers for retina and eye perfusion		500
Strip-chart recorder for spectrophotometer		950
Semi-micro Waring Blendor		120
Sub-Total for D		$ 4,320
E. Indirect costs (15% of A+B+C)	E	$ 3,340
	Total request	$29,910

O.K. L.B.

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	14,500	4,000	3,800	1,000	3,340	26,640
Year 3	--	--	--	--	--	--

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Budget Justification

A. Salaries

The principal investigator plans to spend approximately 50% of his time on this research project over the next two years, if the grant is awarded. The amount of salary requested, however, ($5,000) amounts to only 25% of his present salary. This is in order to keep salary requests to a reasonable figure. From the literature provided us by the Tobacco Research Council, we are not aware of any particular policy on salaries for professional workers. The amount requested for a full-time technician ($9,000) is a figure consistent with Albany Medical College's institutional policy as suggested by the College's Director of Personnel, for an individual holding a Bachelor's degree in Chemistry or Biology. The projected budget for the second year contains a $500 increase in salary for this individual, as acknowledgement for increased experience and proficiency.

B. Consumable Supplies

This category is difficult to itemize in detail, but the figure of $4,500 is one which we find from previous experience to be an approximate amount for a new project where new types of glassware and chemical reagents need to be ordered. The chromatographic separation of Vitamin A aldehyde, alcohol and esters will require some new equipment for column and thin-layer chromatography which we do not presently have on hand and we feel that the figures given are a reasonable estimate of what will be required for the first year.

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Budget Justification

C. Other Expenses

We estimate that our studies on the cat electroretinogram (ERG) will require between 50 and 75 cats and our metabolic studies on rabbit retina will require about 100 rabbits. Present cost of cats are about $16.00 and for rabbits about $6.00 each, totaling about $1200 for the former and $600 for the latter, leaving about $200 of the total $2000 budgeted for animal care for the first year. A local abbatoir has agreed to supply us with freshly excised porcine eyes at 50 cents per eye. At least 50 eyes will be required weekly to obtain sufficient pigment epithelium to make once or twice weekly studies. If 30 weeks are allotted to these studies for the first year at $25.00 per week, the stated figure of $750 is arrived at.

Photographic and illustration work will be done by our Medical Illustrations Department of the College and will include graph drawing, ERG photograph development and mounting, picture taking and mounting for journal papers and 2 x 2 slide preparations for meeting presentations.

Computer analyses and consultation for statistical analyses of biochemical and physiological data may be required beyond the capabilities provided by our Department computer for routine analyses. We feel that the sum of $100 is a modest one for this eventuality.

The travel funds are requested so that the principal investigator may present papers at the annual meetings of FASEB and the Pharmacology Society (ASPET) each of which he is a member of.

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D. Permanent Equipment

The papers cited on the methods for studying cytochrome containing

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enzyme systems in pigment epithelium and those describing the in-vitro retina-optic nerve preparation mention the necessity of using glass distilled water since trace heavy metals (e.g. copper) found in water obtained from our copper Barnstead stills might cause enzyme inhibition and inhibit the function of the retina in these preparations. We do not presently have a source of glass-distilled water and the $600 listed for this purpose is for the smallest and least expensive model we can find to provide us with working quantities.

The Grass photostimulator is needed for the ERG studies on the anesthetized cat and for the retina-optic nerve preparation.

The Dubnoff metabolic shaker is needed as the incubator for the in-vitro metabolic studies in porcine retinal pigment cell suspensions and on isolated rabbit retinae. We recently did some preliminary studies with rabbit retinae using a metabolic shaker borrowed from another laboratory but we were obliged to return it to its original owner recently.

The miscellaneous items of electronic and gas delivery equipment are for ERG and nerve electrodes, the possible neeed for a closed system of respiratory valves for our anesthetized cat preparation.

The Lucite chambers for the isolated rabbit retina-optic nerve and the perfused cat eye preparation will need to be made to order either from our own shop or from an outside source since these are not commercially available. The $500 allowed for this also allows a bit for specialized tubing, valves and reservoirs which may be needed as well.

The Strip-Chart recorder for the Beckman Model DB spectro-photometer is essential for the accurate determination of the spectral cur-

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enzyme systems in pigment epithelium and those describing the in-vitro yes produced by the CO-cytochrome complexes which we hope to find evidence for in our biochemical studies. We have in the past, tried to do these spectral curves manually and find this to be a very time-consuming and inaccurate alternative.

The semi-micro Waring Blendor is needed for making homogenates of the bovine and porcine pigment epithelium.

In summary we have tried, especially with the requests for permanent equipment, to keep our budget in line with our immediate requirements for this project and we will, whenever possible, use our existing equipment, and will borrow from others in the institution where feasible, as, eg in our need for a high-speed centrifuge.

another laboratory but we were obliged to return it to its original owner recently.

The miscellaneous items of electronic and gas delivery equipment

the perfused cat eye preparation will need to be made

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Leonard Procita

Leonard Procita, Ph.D.
Professor & Acting Chairman
Department of Pharmacology

Checks payable to

Mr. Bernard Siegel
Vice President, Business & Finance

Mailing address for checks

Albany Medical College
Albany, New York 12208

Principal investigator

Typed Name Alphonse J. Ingenito, Ph.D.

Signature Alphonse J Ingenito Date 12/30/74

Telephone 518--445-5413
Area Code Number Extension

Responsible officer of institution

Typed Name Eugene H. Horn, Ph.D.

Title Associate Dean

Signature [signature] Date 12/30/74

Telephone (518) - 445 - 5548
Area Code Number Extension

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CURRICULUM VITAE

Alphonse J. Ingenito

The Albany Medical College of Union University
Albany, New York

(518) 445-5413

I. BIRTHPLACE AND DATE: Harrison, New Jersey; October 8, 1932

II. EDUCATION:

1. St. John's University, College of Pharmacy, New York, New York; B.S., 1953 (Pharmacy).
2. Rutgers University, College of Pharmacy, Newark, New Jersey; M.S., 1960 (Pharmacology).
3. University of Wisconsin Medical School,Dept. Pharmacology, Madison, Wisconsin; 1960-62; No degree granted.
4. College of Medicine and Dentistry of New Jersey at Newark, Dept. Pharmacology; Ph.D., 1965 (Pharmacology).

III. PROFESSIONAL EXPERIENCE:

1. Rutgers University, College of Pharmacy. Assistant Instructor in Biological Sciences, Sept. 1958 to June 1960. Laboratory instruction to undergraduate students in Pharmacology and other biological sciences. Research in Pharmacology leading to M.S. degree, concerned with CNS actions of antihistaminic-antiserotonin compounds.

2. University of Wisconsin Medical School, Dept. of Pharmacology. U.S.P.H.S. Pre-Doctoral Trainee, Sept. 1960 to July 1962. Research in Pharmacology in the psychopharmacology program concerned with the functional role of biogenic amines in developing rat and chicken brains.

3. College of Medicine and Dentistry of New Jersey at Newark, Dept. of Pharmacology. U.S.P.H.S. Pre-Doctoral Trainee, Sept. 1962 to July 1965. Lecturing and laboratory instruction to medical and dental students. Research in Pharmacology leading to Ph.D. degree concerned with the role of central biogenic amines in temperature regulation in the rat, and the interactions of various drugs with brain amines and body temperature.

4. Albany Medical College, Dept. of Pharmacology. Instructor in Pharmacology, July 1965 to July 1968. Assistant Professor of Pharmacology, July 1968 to July 1972. Associate Professor of Pharmacology, July 1972 to present. Lecturing and laboratory instruction in Pharmacology to medical students and other health sciences students and in graduate Ph.D. program in Pharmacology. Research with an in-situ brain perfusion technique in cats designed to assess the effects of drugs influencing the peripheral circulation by a central action. Effects of tobacco smoke constituents (nicotine, carbon monoxide, oxides of

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III. PROFESSIONAL EXPERIENCE (cont'd):

nitrogen) on brain and cardiac metabolism and function. Effects of carbon monoxide on the metabolism and function of the visual system (retina, optic nerve and brain).

IV. OTHER PROFESSIONAL EXPERIENCE:

Registered Pharmacist, states of New York and New Jersey.
Employed as a retail pharmacist in New Jersey, 1956 to 1958.

V. SERVICE WITH ARMED FORCES:

U. S. Army, October 1954 to August 1956.
Highest rank attained - private first class.
Occupational duty - hospital pharmacist with evacuation hospital in Korea.
Separation - July 1956. Honorable Discharge - 1962.

VI. PROFESSIONAL AFFILIATIONS:

Member - American Society for Pharmacology and Experimental Therapeutics. since 1970
Committee on Public Affairs, 1973-76
Fellow - American College of Clinical Pharmacology, since 1971
Program Chairman, Annual Meetings, 1973 and 1974
Member - AAAS
Vice President, New York State Society for Medical Research

VII. MARITAL AND DEPENDENCY STATUS:

Married (1962) with 4 children.

Wife: Barbara Joan Steffen
Children and year of birth: Diana Lynn (1963), Jeanne Marie (1964), Carol Ann (1966), James Francis (1973).

VIII. PUBLICATIONS:

Ingenito, A. J. and Bonnycastle, D. D.: Amine Levels in Rat Brain During Cold Acclimation and Following Reserpine and Acute Cold Exposure. The Pharmacologist 6: 194 (1964) (abstract).

Ingenito, A. J. and Bonnycastle, D. D.: On the Relationship Between Drug-Induced Changes in Brain Amines and Body Temperatures of Rats in a Hot Environment. Proc. Can. Fed. Biol. Soc. 8: 28 (1965) (abstract).

Ingenito, A. J.: The Effect of Acute and Prolonged Cold Exposure on the Brain Amine Depleting Action of Reserpine. Arch. int. Pharmacodyn. 166: 324-332 (1967).

Ingenito, A. J. and Bonnycastle, D. D.: On the Relationship Between Drug-Induced Changes in Brain Amines and Body Temperature. Can. J. Physiol. Pharmacol. 45: 723-732 (1967).

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VIII. PUBLICATIONS (cont'd):

Ingenito, A. J. and Bonnycastle, D. D.: The Effect of Exposure to Heat and Cold Upon Rat Brain Catecholamines and 5-Hydroxytryptamine Levels. Can. J. Physiol. Pharmacol. 45: 733-743 (1967).

Ingenito, A. J.: Norepinephrine Levels in Various Areas of Rat Brain During Cold Acclimation. Proc. Soc. Exp. Biol. Med. 127: 74-77 (1968).

Barrett, J. P., Ingenito, A. J. and Procita, L.: A Brain Perfusion Method Adapted for the Study of Drugs Affecting the Peripheral Circulation Through a Central Action. The Pharmacologist 9: 187 (1967) (abstract).

Ingenito, A. J., Barrett, J. P. and Procita, L.: Centrally-Induced Hypotensive Effect of Reserpine Perfused Through the Isolated, In-Situ Cat Brain. Fed. Proc. 27: 757 (1968) (abstract).

Ingenito, A. J., Barrett, J. P. and Procita, L.: Centrally-Induced Cardiovascular Effects of Hydralazine Perfused Through the Isolated, In-Situ Cat Brain. The Pharmacologist 10: 156 (1968) (abstract).

Ingenito, A. J., Barrett, J. P. and Procita, L.: Alpha-methyl-dopa Hypotension: A Centrally-Mediated Effect. Fed. Proc. 28: 737 (1969) (abstract).

Barrett, J. P., Ingenito, A. J. and Procita, L.: A Brain Perfusion Technique Adapted for the Study of Drugs Which May Affect the Peripheral Circulation Through a Central Action. J. Pharmacol. Exp. Therap. 170: 199-209 (1969).

Ingenito, A. J., Barrett, J. P. and Procita, L.: Centrally-Mediated Peripheral Hypotensive Effects of Reserpine and Hydralazine when Perfused Through the Isolated In-Situ Cat Brain. J. Pharmacol. Exp. Therap. 170: 210-220 (1969).

Ingenito, A. J., Barrett, J. P. and Procita, L.: Central Autonomic Actions of Nicotine: Effect of Central Deafferentation. Fed. Proc. 29: 741 (1970) (abstract).

Katz, N. L., Ingenito, A. J. and Procita, L.: Ryanodine-Induced Contractile Failure of Skeletal Muscle. J. Pharmacol. Exp. Therap. 171: 242-248 (1970).

Barrett, J. P., Ingenito, A. J. and Procita, L.: Influence of the Carotid Sinus on the Centrally-Mediated Cardiovascular Effects of Angiotensin II. J. Pharmacol. Exp. Therap. 176: 692-700 (1971).

Ingenito, A. J., Barrett, J. P. and Procita, L.: A Centrally-Mediated Peripheral Hypotensive Effect of Alpha-Methyldopa. J. Pharmacol. Exp. Therap. 175: 593-599 (1970).

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VIII. PUBLICATIONS (cont'd):

Ingenito, A. J., Barrett, J. P. and Procita, L.: An Analysis of The Effects of Nicotine on the Cerebral Circulation of an Isolated, Perfused In-Situ Cat Brain Preparation. STROKE-A Journal of the Cerebral Circulation 2: 67-75 (1971).

Ingenito, A. J., Barrett, J. P. and Procita, L.: Direct Central and Reflexly Mediated Effects of Nicotine on the Peripheral Circulation. Europ. J. Pharmacol. 17: 375-385 (1972).

Ingenito, A. J. and Procita, L.: Augmentation of Pressor Response to Common Carotid Occlusion by α-Methyldopa. A Paradox. Fed. Proc. 30: 676 (1971) (abstract).

Ingenito, A. J. and Procita, L.: Cigarette Smoke Induced Formation of Methemoglobin in Blood. Fed. Proc. 32: 805 (1973) (abstract).

Procita, L. and Ingenito, A. J.: Comparison of the Cardiovascular Effects of Some Gaseous Components of Tobacco Smoke. Fed. Proc. 32: 805 (1973) (abstract).

Ingenito, A. J., Fiedler, P. C. and Procita, L.: Effect of Carbon Monoxide on Adenine Nucleotides in Brain and Heart. The Pharmacologist 15: 259 (1973) (abstract).

Ingenito, A. J., Fiedler, P. C. and Procita, L.: Negative Inotropic Action of Carbon Monoxide on the Isolated Isovolumic Heart with a Hemoglobin-Free Perfusate. Fed. Proc. 33: 503 (1974) (abstract).

Ingenito, A.J., Ryon, J.J. and Procita, L.: Carbon Monoxide-Nicotine Interactions on the Isolated, Perfused, Isovolumic Rabbit Heart. Fed. Proc. 1975 (abstract in the press).

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An Analysis of the Effects of Nicotine on the Cerebral Circulation of an Isolated, Perfused, in situ Cat Brain Preparation

BY ALPHONSE J. INGENITO, PH.D., JAMES P. BARRETT, PH.D., M.D.,* AND LEONARD PROCITA, PH.D.

Abstract: *An Analysis of the Effects of Nicotine on the Cerebral Circulation of an Isolated, Perfused, in situ Cat Brain Preparation*

■ To determine the effects of nicotine HCl on the cerebral circulation of the cat, without the complicating actions of the drug at other sites in the body, the drug was perfused at concentrations of 1, 10 and 100 μg/ml through a vascularly isolated, perfused in situ cat brain preparation. Cats having selective section of cranial nerves 9, 10, 11 and 12 and the cervical sympathetic trunks comprised various experimental groups. Nicotine was also perfused through an isolated, denervated hindlimb of the same cat for comparative purposes. Nicotine caused only a mild and transient vasoconstriction of the cerebral circulation, mediated primarily by stimulation of the superior cervical ganglia, and a small direct cerebral vasoconstrictor component. An acute tolerance to the cerebral vasoconstrictor effects of repeated increments of nicotine was also observed. The cerebral vasoconstrictor effect of nicotine was diminished in the presence of intact vagi, suggesting a cerebral vasodilator role for these nerves. In contrast, the effect of nicotine on the denervated hindlimb vasculature was a weak but sustained vasodilation. The mechanism of action of nicotine on the cerebral circulation is discussed along with the relevance of these findings to the potential health hazards of tobacco smoking in individuals with cerebrovascular insufficiency.

ADDITIONAL KEY WORDS
innervation of cerebral circulation
smoking and cerebral ischemia
nicotine tolerance
cerebral vasoconstriction
vagal cerebral vasodilation
nicotine and hindlimb circulation

Introduction

□ Despite occasional speculation as to the relationship between cerebrovascular disease and smoking[1-3] and the suggestion that smoking be discouraged in patients with cerebral vascular insufficiency,[4] relatively little is known of the effects of smoking or nicotine on the cerebral circulation.[5, 6] Some studies in man have shown little or no effect of cigarette smoking or intravenously injected nicotine (in "smoking" doses) on cerebral blood flow and cerebrovascular resistance (CVR).[7-9] Larson et al.[5] cite a number of older studies on experimental animals in which cerebral blood flow was measured directly or by visual observation of exposed cortical vasculature following tobacco smoke inhalation or systemically injected nicotine. In most of these studies the action of nicotine was either vasodilator or

From the Department of Pharmacology, Albany Medical College, Albany, New York, 12208.

*Present address: Department of Medicine, Germantown Hospital, Philadelphia, Pennsylvania.

Reprint requests should be sent to Dr. Alphonse J. Ingenito, Department of Pharmacology, Albany Medical College, Albany, New York, 12208.

Supported by a grant-in-aid from the American Medical Association Education and Research Foundation, Committee for Research on Tobacco and Health.

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#1012-MITCHELL

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

December 27, 1974

Grant Application No. 1012

PHARMACOLOGY

To: The committee comprising Drs. Gardner, Meier and Wyatt

Subject: J. Andrew Mitchell, Assistant Professor, Ph.D., Dept. Anatomy, Wayne State University, School of Medicine, Detroit, Mich.
New application No. 1012
"A Study of the Effects of Nicotine on Gestation in the Rat with Particular Reference to Implantation and the Time of onset of Parturition"

History

An informal inquiry was handled as Case No. 237: the Executive Committee voted to encourage formal application.

Request

Application No. 1012 requests $6,887 for the first year and $4,945 for the second year.

Documents Submitted (attached)

Application dated September 5, 1974 (29 pages) including C.V.'s of Dr. Mitchell and graduate student D. R. Garris.

D.S.

DS:wg
Encls.

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#1012

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

SEP 2 4 1974

Application For Research Grant

Date: 9/5/74

1. Name of Investigator(s): (include Title and Degrees) J. Andrew Mitchell
Assistant Professor
Ph.D.

2. Institution & Address: Wayne State University
School of Medicine
Department of Anatomy
540 East Canfield Street
Detroit, Michigan 48201

3. Short Title of Project:

A Study of the Effects of Nicotine on Gestation in the Rat with Particular Reference to Implantation and the Time of Onset of Parturition

4. Proposed Starting Date: January 1, 1975

5. Anticipated Duration of this Specific Study: 2 years

6. Brief Descripton of Objectives or Specific Aims:

Considerable evidence indicates that nicotine adversely affects pregnancy in the rat. Among such disruptive effects are increased frequency of fetal resorption, reduced litter size and weight, and delayed parturition. The primary objective of the experiemnts embodied in this proposal is to gain a greater understanding of the means by which nicotine exerts disruptive effects on the normal course of pregnancy, and more particularly, how the chief alkaloid of tobacco reduces litter size and delays parturition. To this end, a variety of parameters will be measured to assess reproductive efficiency, time of implantation, fetal growth and development, and normalcy of parturition in nicotine treated vs. control rats. Several experimentally testable hypothesis are proposed. Specifically, experiments will be conducted to determine if nicotine induced reduction in litter size and delay in parturition are related to alterations in uterine sensitivity to implantation. The time of onset, magnitude and duration of sensitivity to decidualization will be compared in treated and control rats. In addition, the effects of nicotine on duration of gestation will be determined by an accurate measurement of the time of parturition.

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7. Give a Brief Statement of your Working Hypothesis: please see page 4 of attached proposal.

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

Please see page 7 of attached proposal.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

A well equipped endocrinology laboratory and two animal rooms are available. In addition, complete histology and veterinary services are available as well as computer and photographic facilities.

10. Additional Requirements: None

11. Biographical sketches of all principal and professional personnel (append)

appended

12. List of publications: (Five most recent as pertinent) (append)

appended

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13. Budget (1st year)

	% time	Amount
A. Salaries (Personnel by names)		
Professional		
J. A. Mitchell Ph.D.	35	REDACTED
Technical		
Graduate Students:		
1) D. R. Garris	80	REDACTED
2) to be determined	80	REDACTED
Sub-Total	R	
B. Consumable Supplies (list by categories)		2000.00
Rat food & litter, animal care fee		1000.00
General reagents		200.00
Glassware, general lab supplies		500.00
Animals		600.00
Sub-Total		
C. Other Expenses (itemize)	2300.00	4300.00
Publication cost		150.00
Page cost		60.00
Photography		150.00
Reprints		250.00
Computer Fee		
Sub-Total		
D. Permanent Equipment (itemize)	610.00	4910.00
Kymograph (research quality)		360.00
Surgical instruments		200.00
Roller-Smith Micro Balance		380.00
Animal cages		300.00
E. Overhead (15% of A+B+C)	1240.00	6150.00
Total	736.50	6886.50

O.K. J.B.

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	R	1500.00	800.00	0	645.00	4945.00
Year 3	–	–	–	–	–	–

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature J. A. Mitchell
Director of Project J.A. Mitchell, Ph.D.
577-1061 Telephone

Signature Thomas J. Curtin
Business Officer of the Institution Dr. T.J. Curtin

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
A Study of Intrauterine Oxygen Tension in the Guinea Pig with Particular Reference to the Availabiltiy of Oxygen at the Time of Implantation	Wayne State University General Research Support Grant	$3,651.00	1973-1975

Pending

Department of Anatomy will provide additional funding up to 20% of the total grant received

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R: REDACTED MATERIAL

CURRICULUM VITAE

J. Andrew Mitchell

Date of Birth: REDACTED

Place of Birth: Dallas, Texas

Marital Status: REDACTED

Education:

B.S.	1963	Southern Methodist University, Dallas, Texas Dr. John L. McCarthy, Research Advisor Major: biology, Minor: chemistry, philosophy
Ph.D.	1969	University of Kansas, Lawrence, Kansas Dr. Jerome M. Yochim, Dissertation Advisor Major: physiology, Minor: biochemistry

Positions:

1962-1964	Teaching Assistant in Physiology, Biology and Microbiology
1966-1968	Teaching Assistant in Physiology, Biochemistry and Advanced Endocrinology
1969-1972	Post-Doctoral under Dr. Vaughn Critchlow, Baylor College of Medicine, Department of Anatomy, Houston, Texas
1972-	Assistant Professor of Anatomy, Department of Anatomy, Wayne State University School of Medicine

Awards & Fellowships:

1966-1967	University of Kansas Research Assistantship
1967-1968	University of Kansas Research Assistantship
1967	National Science Foundation Summer Graduate Traineeship
1968	William King Candlin Award in Physiology
1969-1971	Public Health Service Postdoctoral Research Fellowship

Memberships:

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Foreign Languages:

Reading knowledge of French and German

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Research Interests:

Physiological and biochemical aspects of nidation
Photoperiod effects on reproductive phenomena
Regulation of ACTH and growth hormone secretion
Neural regulation of circadian ACTH release
Neural regulation of gonadotrophin secretion

Publications:

McCarthy, J.L., A.L. Brodsky, J.A. Mitchell and R.F. Herrscher 1964 Thin-Layer Chromatography of Adrenal Corticoids: Observations on Changes in Mobilities, Analytical Biochemistry 8:164-170.

Mitchell, J.A. and J.M. Yochim 1968 Relation of HCG-Induced Ovulation to the Production of Prolonged Diestrus in the Adult Rat, Endocrinology, 82:1142-1148.

Mitchell, J.A. and J.M. Yochim 1968 Measurement of Intrauterine Oxygen Tension in the Rat and Its Regulation by Ovarian Steroid Hormones, Endocrinology, 83:691-700.

Mitchell, J.A. and J.M. Yochim 1968 Intrauterine Oxygen Tension During the Estrous Cycle in the Rat: Its Relation to Uterine Respiration and Vascular Activity, Endocrinology, 83:701-705.

Yochim, J.M. and J.A. Mitchell 1968 Intrauterine Oxygen Tension in the Rat During Progestation: Its Possible Relation to Carbohydrate Metabolism and the Regulation of Nidation, Endocrinology, 83:706-713.

Yochim, J.M. and J.A. Mitchell 1970 Influence of Environmental Lighting on Decidualization in the Rat, Endocrinology, 87:465-471.

Mitchell, J.A. and J.M. Yochim 1970 Influence of Environmental Lighting on Duration of Pregnancy in the Rat, Endocrinology, 87:472-480.

Mitchell, J.A., M. Hutchins, W. Schindler and V. Critchlow 1972 Plasma Growth Hormone Levels in Rats with Increased Naso-Anal Lengths Due to Hypothalamic Surgery, Neuroendocrinology, 10:31-45.

Mitchell, J.A., M. Hutchins, W.J. Schindler and V. Critchlow 1973 Increases in Plasma Growth Hormone Concentration and Naso-Anal Length in Rats Following Surgical Isolation of the Medial Basal Hypothalamus, Neuroendocrinology, 12:161-173.

Mitchell, J.A., Ependymal Tanycytes and Anterior Pituitary Function (a review submitted for publication)

Belsare, D.K., J.A. Mitchell and V. Critchlow 1974 Neural Structures that Trigger Ovulation in the Rat, Journal of Reproduction and Fertility, 38: 242-243.

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Abstracts:

Mitchell, J.A. and J.M. Yochim 1969 Influence of Photoperiod on the Duration of Gestation and the Time of Parturition in the Rat, The Anatomical Record, 163: 230.

Mitchell, J.A. and J.M. Yochim 1969 The Influence of Photoperiod on the Onset, Duration and Magnitude of Uterine Sensitivity to Decidualization in the Rat, The Anatomical Record, 163: 317.

Mitchell, J.A., D.K. Belsare, M. Hutchins, W.J. Schindler and V. Critchlow 1971 Increases in Growth and Plasma Growth Hormone Levels Following Surgical Isolation of Medial Basal Hypothalamus in Female Rats. The Endocrine Society, Program of the 53rd Meeting, P. A-89, Paper No. 79.

Mitchell, J.A., D. Belsare, M. Hutchins, W. Schindler and V. Critchlow 1972 Increases in Growth and Plasma Growth Hormone Levels Following Horizontal Cuts in the Ventromedial Hypothalamus in Female Rats. The Anatomical Record, 172: 368.

Yochim, J.M. and J.A. Mitchell 1970 Influence of Environmental Lighting on Decidualization in the Rat, Endocrinology, [illegible] 471.

Lighting on Duration of Pregnancy in the Rat, Endocrinology, 87:472-480.

Increases in Plasma Growth Hormone [illegible]

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Abstracts:

Invited Lecturer:

Mitchell, J.A. and J.H. Vocmar 1969 Influence of Photoperiod on the

C.S. Mott Center Colloquium "Endocrine Functions of the Hypothalamus."
Lecture Title: "Structure-Function Relationships in the Hypothalamus"
February 7, 1974.

Mitchell, J.A. and J.H. Vocmar 1969 The Influence of Photoperiod on

William Beaumont Hospital Neurology Residency Program
"Basic Concepts in Neuroendocrinology"
March 1, 1974.

Mitchell, J.A., D.K. Belsare, M. Hutchins, W.I. Schindler and V. Critchlow

Invited Panel Discussant: in Gross and Plasma Growth Hormone Levels

Society for Neuroscience - Michigan Chapter
"Neuroanatomical Substrates of Neuroendocrine Intergration"

Meetings Attended: D. Belsare, M. Hutchins, W. Schindler and V. Critchlow

7th Annual Scanning Electron Microscope Symposium
Chicago, Illinois, April 8 - 11, 1974.

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R: REDACTED MATERIAL

CURRICULUM VITAE

Name: David R. Garris

Address:
Department of Anatomy
Wayne State University
School of Medicine
G. H. Scott Hall of Basic Medical Sciences
540 E. Canfield
Detroit, Michigan 48201

Birth Date: REDACTED

Marital Status: REDACTED

Education:

University	Major	Minor	Degree
Eastern Michigan University Ypsilanti, Michigan 1969-1973	Biology	Chemistry	B.S.
Wayne State University Detroit, Michigan 1973-1974	Anatomy	Physiology Biochemistry	

Memberships:

REDACTED

Awards and Fellowships:

National Science Foundation COSIP Student Department of Chemistry 1973 — Eastern Michigan University

National Science Foundation COSIP Student Department of Biology 1973 — Eastern Michigan University

Wayne State University Graduate Teaching Assistantship — 1973-1974

Teaching Experience:

Teaching Assistant (EMU) — Histology

Teaching Assistant (WSU) — Anatomy, Neuroanatomy

Meetings Attended:

The Society for the Study of Reproduction Ottawa, Canada — August, 1974

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Name: David R. Garris

Address: Department of Anatomy
Wayne State University
School of Medicine
G. H. Scott Hall of Basic Medical Sciences
540 E. Canfield
Detroit, Michigan 48201

Birth Date: October 28, 1951

Marital

A Study of the Effects of Nicotine on Gestation in the Rat with Paricular Reference to Implantation and the Time of Onset of Parturition.

Educat

Easter	Biology	Chemistry	B.S
Wayne State University Detroit, Michigan	Anatomy	Physiology Biochemistry	

J. A. Mitchell

Wayne State University

School of Medicine

Department of Anatomy

National Science Foundation COSIF Student Department of Biology 1972

Eastern Michigan University

Teaching Assistant (EMU) Histology

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1. Present State of Knowledge

The results of numerous studies have established that nicotine may interfere with the normal course of pregnancy in the rat. It has been claimed that the administration of nicotine to pregnant rats results not only in reduced litter size (20-22, 38, 40, 42) and litter weight (2-5, 15, 18, 19, 25, 26) but also in increased frequency of stillbirths (4, 11, 22) and infant mortality (2-4, 10, 11, 14, 20, 32, 34).

Among the most systematic and informative studies of the actions of nicotine on pregnancy are those of Becker et al., (2-5). When twice daily sc injections of 3.0 or 5.0 mg/kg of nicotine were administered throughout pregnancy, the onset of parturition was delayed as much as 3 to 5 days, the mean delay resulting from the higher dose being 2 days (4). Also, the young weighed less and were retarded in development relative to controls.

In another study, Becker et al , (5) again employed the 5 mg/kg dose to prolong gestation. However, for comparison non-nicotine treated rats were given chorionic gonadotrophin in order to prolong pregnancy. Although equivalent delays in parturition were achieved in the two groups (all were delivered by Ceasarean section of Day 21, 22, 23, or 24), the characteristic of the young were markedly different. Young born following hormonally prolonged gestation evidenced the typical post-maturity syndrome, i.e., greater body weight and development compared with

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neonates born at term. In contrast the neonates of nicotine treated rats, though subjected to an equivalent delay in parturition, were markedly lighter in weight and retarded in development. More recently, Hudson and Timiras (17) investigated the effects of nicotine on reproductive capacity and length of gestation, as well as on litter size and viability. Injection of 5 mg/kg of nicotine, administered sc twice daily throughout pregnancy, prolonged gestation by 2-3 days; the most protracted pregnancy was of 27 days duration. However, in contrast with the results of Becker et al., (4, 5) birth weights were not statistically different between treated and control rats. Furthermore, no mention was made of retarded development.

Birth weights of nicotine treated rats did not differ from those of control rats in spite of prolonged gestation, which suggests that either the rate of fetal growth was reduced throughout gestation, or that the rate of growth was normal but slow in starting. The failure or prolonged gestation in nicotine treated rats to result in larger and more mature neonates favors the latter interpretation and suggests that nicotine delays implantation. A delay in uterine sensitivity would produce a delay in implantation which in turn could be reflected as a delay in parturition. Such a delay in nidation would be consistent with the birth of young of normal weight and development as reported by Hudson and Timiras (17).

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However, other explanations for the observed delay in parturition are possible. The prolongation in gestation reported by Becker et al. (4, 5) was associated with reduced fetal bulk. A reduction in fetal mass may contribute toward a delay in parturition (29). It is also possible that nicotine reduces fetal mass by reducing the magnitude of uterine sensitivity to the blastocyst, thereby diminishing litter size. Reduction in litter size following administration of nicotine has been observed (19, 33, 35). Another possibility is that nicotine may delay the onset of parturition, not by delaying implantation but by retarding the rate of fetal growth by suppressing maternal food intake (4). The proposed experiments are designed to discriminate between these various possibilities.

2. Specific Objectives

The primary objective of the experiments embodied in this proposal is to gain a greater understanding of the means by which nicotine exerts disruptive effects on pregnancy in the rat, and more particularly, how the alkaloid brings about reduction in litter size and delay in parturition. The following experimentally testable hypotheses are proposed:

I. That nicotine reduces uterine sensitivity to implantation of the blastocysts.

II. That nicotine delays the normal time of onset of uterine sensitivity to implantation of the blastocysts.

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III. How That the nicotine induced delay in time of parturition in rats is a reflection of the delay in the time of implan- reported tation of the blastocyst. was associated with reduced fet a The following is a brief resume of why it is reasonable to assume that nicotine may alter endometrial physiology: nic Ample evidence has been marshalled to support the long held contention that uterine sensitivity to decidualization is a reliable model for studying uterine receptivity to the rat blastocyst (13). Because induction of the decidual cell reaction has proved so useful in investigating the process of nidation itself, considerable work has been directed toward determining the physiological and metabolic conditions which are necessary to support the response.

va Not only has the temporal course of uterine sensitivity to decidualization been worked out in detail (38), but the hormonal balance requisite for optimal deciduoma formation has been established (37). It is now known that the magnitude of the decidual response as well as the timing of sensitivity is regulated by an estrogen-progesterone interplay. Although a decidual response may be evoked in the presence of progesterone alone, it is submaximal in magnitude; estrogen must also be present for maximal responsivity to occur. Although estrogen potentiates the uterine sensitizing effect of progesterone, it also curtails the period during which a maximal decidual reaction can be evoked (38).

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Therefore, whereas progestogens render the endometrium receptive to the blastocyst, estrogen regulates the magnitude and duration of receptivity. The regulation of the secretion of both of these ovarian steroid hormones is, of course, under the control of the anterior pituitary and thereby ultimately regulated by the hypothalamus.

The process of nidation is dependent on the proper synchronization of ovulation and sensitivity of the endometrium to the presence of the blastocysts (26). Furthermore, like decidualization, the development and maintenance of uterine sensitivity to implanation is dependent upon the proper balance of ovarian steroids (13), the secretion of which is, at least partially, regulated by lutenizing hormone (LH) and prolactin from the anterior pituitary (1). Recently, studies by Blake, et al. (6-8) have established that nicotine may alter the timing of the ovulatory surge of LH from the anterior pituitary via action on the hypothalamus. Although the study was confined to the effects of nicotine on the timing of the critical period for LH release in ovulation, the findings raise the possibility that nicotine may similarly alter LH release not only prior to ovulation, but perhaps also during early pregnancy. Similarly, nicotine administration inhibits the proestrous surge of prolactin (9). Thus, it is possible that appropriately timed administration of nicotine may alter LH and/or prolactin release in such a

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Therefore, whereas progestogens render the endometrium manner as to impair the delicate balance of ovarian steroids required to maintain uterine receptivity to the blastocyst, and thereby delay and/or impair uterine sensitivity to implantation.

In addition to the possibility that nicotine may alter the course of nidation via its action on the CNS it may also influence implantation via local uterine effects. For example, it has been suggested that the site of blastocyst invasion is influenced by the abundance of intrauterine vasculature, implantation occurring preferentially near an area of increased intrauterine pO_2 (39). Since nicotine is known to be a vaso-active substance, it is possible that the alkaloid may influence blastocyst attachment by altering the endometrial vasculature, thereby modifying oxygen availability.

That nicotine may exert an influence in implantation via action of the blastocyst itself as well as on the endometrium is suggested by the study of Fabro and Sieber (16). Following iv injection of G-(^{3}H)-nicotine into rabbits, radioactivity was detected in the endometrium, uterine fluid, and blastocyst. The data suggest that nicotine may be taken up prior to implantation by the blastocyst.

3. Experimental Designs

A. Treatments

Hypotheses I & II, that nicotine reduces uterine sensitivity to implantation and/or delays the onset of such sensitivity will be tested in the following ways:

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1) Comparing the time of onset, duration and magnitude of uterine sensitivity to decidualization in control vs. nicotine treated rats.

2) Estimating of the time and frequency of implantation in control vs. nicotine treated rats by, a) direct visual detection of early implant sites, and b) determination of the time during which free blastocysts can be recovered from the uterus by flushing.

Hypotheses III, that the delay in parturition observed in nicotine treated rats reflects the delay in the time of implantation of the blastocyst, will be tested by accurately measuring the time of parturition in control vs. nicotine treated rats.

The morning on which spermatozoa are found in the vaginal lavage will be designated Day 0 of pregnancy. Insemination will arbitrarly be assumed to have occurred the previous midnight. For each experiment, inseminated rats will be assigned randomly to one of two groups: nicotine treated and control. Rats in the treated group will be assigned randomly to one of 3 dosages of nicotine: 2, 6 and 10 mg/kg of nicotine per day. The alkaloid will be injected sc twice daily in 2 equal doses, the first administered between 8:00-9:00 am, the second between 5:00-6:00 pm. The 2 mg/kg dose, while pharmacological in the rat, is comparable to that received by a heavy smoker according to the studies of Wolff et al., (36).

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In addition to studying the effects of different dosages of nicotine on the parameters selected, the effects of duration of treatment will be studied. In one series of experiments, the various dosages of nicotine will be administered only during the initial 8 days of pregnancy or pseudopregnancy. In another series, the nicotine treatment will be continued throughout the duration of pregnancy. In addition, other dose levels and schedules may be used, as suggested by the experimental results obtained.

B. Reagents

The nicotine to be used will be of the purest grade (Eastman Kodak) and prepared as a 2% stock solution in 0.85% NaCl solution for dilution as necessary to prepare the proper dosage. The stock solution will be prepared at 2-3 day intervals, stored in amber glass bottles and refrigerated.

Control rats will receive equivalent volumes of 0.85% NaCl solution via sc injections administered on the same schedule as the nicotine treated rats. Otherwise, treated and control animals will be cared for in identical fashion.

4. Materials and Methods

A. Animals, Environment and Routine Procedures

Mature virgin female rats of the Holtzman strain, 200-300 g body weight, will be maintained under controlled environmental conditions with respect to room temperature

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(20 - 25°C), humidity (40-60%) and photoperiod (14 hr. light, 10 hr. darkness). Illumination will be provided by standard General Electric "cool white" fluorescent lights (color temperature 4000 - 5000 K) regulated by an automatic timer. The intensity of illumination at the levels of the cages will range from 430 to 1180 lumens/m^2. Animals will be housed 1/cage and provided free access to Purina Lab Chow and water. Vaginal smears will be recorded daily for at least 3 complete estrous cycles prior to breeding or induction of pseudopregnancy.

Pseudopregnancy will be induced by mechanical stimulation of the uterine cervix with a glass rod during proestrus and estrus. Day 0 of pseudopregnancy will be defined as the last day of vaginal cornification prior to the onset of the luteal phase; Day 1 will be the day on which the vaginal smear is predominately leukocytic.

B. Measurement of Time of Onset of Parturition

The time of onset of parturition will be measured as previously described (24). On the 19th day of pregnancy, each rat will be transferred to a special cage arrangement which permits the determination of the time of onset of parturition with no disturbance to the animal (Fig. 1). A standard wire-bottom rat cage (7" wide, 9" long & 7" high) will be provided with an aluminum floor which slopes to a 1" x 2" opening at the rear of the cage. Below the opening, chart paper will be moved at a known rate of speed by a

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horizontally arranged kymograph. During parturition, light, amnionic fluid and blood will flow through the hole to drop below and stain the moving chart paper. Be measuring the position of the initial stain, the time of onset of parturition can be calculated within ± 15 minutes. Direct observations have confirmed that this procedure provides a good index of the time of onset of delivery.

Treatment of Data:

Though the apparatus for recording delivery will allow determination of parturition to within ± 15 minutes, mean times of delivery will be calculated for 6 hour intervals: all deliveries within ± 3 hours of the plotted time will be pooled to produce the plotted value. The results will be calculated as the percent of the total experimental population which delivered every 6 hours during Days 21-24 (or whatever span of time is appropriate for the experimental results) of gestation. Differences of less than 20% between comparable experiments will be arbitrarily considered to be of borderline biological significance. To determine the statistical significance of the data, the mean time of delivery will be calculated for each experiment, and analysis of variance will be performed following a confirmation of homogeneity of variance.

C. Induction of Decidualization

Deciduomata will be induced by surgical traumatization as previously described (40). In brief, each rat will be

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laparotomized by a mid-line incision and one or both uterine horns will be traumatized by inserting a knife-tipped needle into the uterine lumen by way of a small incision placed near the junction of the horn and the cervix. The blade will be pushed into the lumen and completely to the ovarian end of the uterus. The knife edge will be pressed against the entire length of the antimesometrial surface of the uterus as it is withdrawn.

Decidualization will be induced in each rat during one of 6 times: Day 2 midnight; Day 3 noon or midnight; Day 4 noon or midnight; Day 5 noon. Five days following uterine traumitization, the rats will be weighed and sacrificed by ether asphyxiation; the uterine cornua will be removed, placed on moist bibulous paper, and trimmed of adhering fat and mesentery. The cornua will be separated and weighed on a torsion balance to the nearest 0.5 mg.

Treatment of Data:

Decidual sensitivity will be expressed in 2 ways: as mg/cornu, and as percent of an arbitrary average response to trauma during the period of maximal sensitivity in normal rats (38). Under these conditions, 100% sensitivity = 2000 mg/cornu. To simplify the interpretation of results, the "onset" of decidual sensitivity will be defined as responses between 40 and 80% of maximal. Statistical analysis of the data will be performed using the one-way analysis of variance.

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5. Additional Experiments and Observations

1) Changes in timing and frequency of implantation

A. Recovery of Blastocysts

As an additional test of the ability of nicotine to alter the time of implantation, the rate of recovery of blastocysts from the uterus during the preimplantation period by saline flushings will be determined. Normally, under the conditions of the present experiments, by early Day 5 nidation has proceeded to a point that blastocysts can no longer be flushed from the uterus. Therefore, a delay in implantation will be suggested if unattached blastocysts are obtainable from nicotine treated rats at significantly later times than in control rats.

An another indication of whether or not nicotine administration results in delayed nidation, the mesometrial-antimesoemtrial width of the implantation sites will be measured on Day 8 of treated and control rats. Such measurements will provide an approximate indication of when implantation occurred (30).

B. Early visual Detection of Implantation

The benzyl-benzoate clearing technique described by Orsini (27) will be used to render implantation sites visible. After fixing, dehydrating, bleaching and clearing, rat uteri illuminated by oblique light reveal nidation sites as early as 5 days and 9 hours following insemination (28). A delay

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in implantation will be indicated if nidation sites are not visible in nicotine treated rats until significantly later times than in control rats.

2) Changes in Birth Weight and Growth, Placenta Weight, the Frequency of Resorption, and Maternal Food Intake.

A. Birth Weight

To determine whether or not reduction of fetal mass occurs, and if so, by what mechanism(s) it takes place, a variety of experiments and observations will be performed. Following delivery, each pup will be weighed, sexed, measured for length and examined for the degree of development and presence of congenital defects. The total litter and mean neonate weights will be computed and percentage of live births noted.

B. Development

Neonates will be relaxed with ether anesthesia and the naso-anal length measured to the nearest mm with vernier calipers. In addition, the stages of development of each newborn will be assessed using the method of Christie (12). This method requires the careful examination of each newborn for specific morphological characteristics, the presence or absence of which permits an estimation of the stage of development with an accuracy of $\pm$ 1/2 day.

C. Growth

As an additional indicator of growth, rate of fetal bone growth will be assessed using procion dyes. The procion dyes

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to be used are derivatives of mono-chlorotriazinyl, are water soluble, and react irreversibly with osseous tissue. Since such dyes do not dissolve during the histological preparation of stained bone, they act as indicators of bone growth (26, 31).

Although the details of timing, dosage, etc., remain to be determined, in general, the method will consist of an initial iv injection of Procion Brilliant Red II-8BS, followed at an appropriate interval by an injection of Procion Brilliant Purple H-3Bs. Shortly after birth, and following measurement of other parameters, the femurs of each neonate will be dissected free, cleaned, weighed, measured for length, and fixed for histological examination. Following decalcification and sectioning, the distance between the red and purple dye lines (resulting from the administration of Procion Brilliant Red and Purple, respectively), will be measured with an ocular micrometer. The rate of femur growth during the interval between dye injections will be computed. Three measurements will be made on each femur and the mean distance between dye lines determined for each. In an effort to eliminate biased measurements, the identity of each specimen will be coded so as to prevent the observer from knowing the treatment administered. All indices of growth, ie., naso-anal length, body weight, femur length and weight, as well as rate of femur growth, will be subjected to appropriate statistical analysis to determine if there are differences between the experimental and control groups.

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D. Placental Growth

The effects of nicotine on placental growth will be determined and morphologic evidence of altered placental function will be sought. In randomly selected animals, (d. 31), direct visual observations will be made to detect the onset of labor. Following delivery of the first pup, the mother will be anesthetized and the remainder of the litter delivered by Caesarean section in order to obtain intact placentae. Such placentae will be cleaned, blotted dry, and weighed. Placental diameter will be measured with vernier calipers, prior to fixation for histological examination.

E. Frequency of Resorption

To determine if nicotine increases the frequency of fetal resorption, each mother rat will be sacrificed by ether anesthesia and the uteri removed, cleaned and weighed. The number and distribution of implantation sites will be determined. The frequency of resorption will be computed for each litter by comparing the number of young in the litter vs. the number of implantation sites.

F. Changes in Maternal Food Intake.

In order to determine if nicotine administration results in reduced maternal food intake, food will be provided in spill proof containers and daily food and water consumption will be measured. Should nicotine treatment be associated with a significant reduction in food consumption, paired feeding

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D. Placental Growth

experiments will be initiated to determine what effect, in any, and equivalent reduction of food intake exerts on fetal growth and the time of parturition in control rats. Controls will be paired randomly with nicotine treated rats inseminated on the same evening. Food intake of nicotine treated animals will be measured daily, and the paired control rat provided with only an equivalent quantity of food. Paired animals will be observed for time of parturition; litters and post-parturient mothers will be subjected to the standard series of measurements to determine if the effects of nicotine are possibly brought about by reduced caloric intake.

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experiments will be initiated to determine what effect, in any and equivalent reduction of food intake exerts on [illegible] growth and the time of parturition in control rats. Controls will be paired randomly with nicotine treated rats inseminated on the [illegible] Food intake of nicotine treated animals will [illegible] and the paired control rat [illegible] only [illegible] food. Paired animals will be observed for time of parturition [illegible]

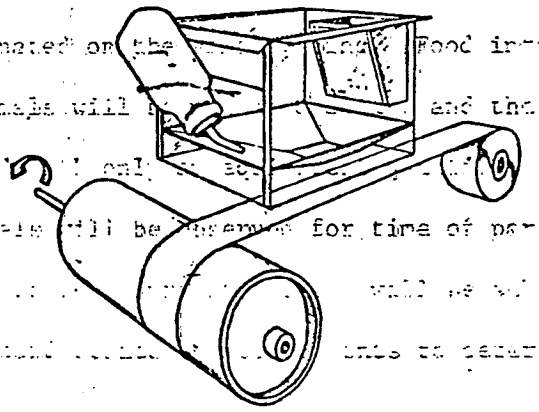

Fig. 1. Apparatus used to determine time of onset of parturition in the rat.

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References

1) Armstrong, D. T. Luteotropic roles of prolactin and luteinizing hormone in the rat, in Progress in Endocrinology, Excerpta Medica International Congress Series No. 184, pp. 89-97, 1968.

2) Becker, R. F., and J. E. King Studies on nicotine absorption during pregnancy. II. The effects of acute heavy doses on mother and neonates. Am. J. Obst. & Gynec. 95: 515-522, 1966.

3) Becker, R. F., and J. King Effects of nicotine absorption in rats during pregnancy. Science 154: 417-418, 1966.

4) Becker, R. F., C. R. D. Little, and J. E. King Experimental studies on nicotine absorption in rats during pregnancy. III. Effect of subcutaneous injection of small chronic doses upon mother, fetus, and neonate. Am. J. Obst. & Gynec. 100: 957-968, 1968.

5) Becker, R. F., J. E. King, and C. R. D. Little Experimental studies on nicotine absorption during pregnancy. IV. The postmature neonate. Am. J. Obst. & Gynec. 101: 1109-1119, 1968.

6) Blake, C. A., R. J. Scaramuzzi, R. L. Norman, S. Kanematsu, and C. H. Sawyer Effect of nicotine on the proestrous ovulatory surge of LH in the rat. Endocrinology 91: 1253-1258, 1972.

7) Blake, C. A., R. J. Scaramuzzi, R. L. Norman, S. Kanematsu, and C. H. Sawyer Nicotine delays the ovulatory surge of luteinizing hormone in the rat. Proc. Soc. Exp. Biol. Med., 141: 1014-1016, 1972.

8) Blake, C. A. Parallelism and divergence in follicle stimulating hormone (FSH) and luteinizing hormone (LH) release in nicotine treated rats. Program of the 6th Annual Meeting of the Society for the Study of Reproduction, No. 34, p. 43, 1973.

9) Blake, C. A., R. Norman, R. Scaramuzzi and C. H. Sawyer Inhibition of the proestrous surge of prolactin in the rat by nicotine. Endocrinology 92: 1334-1338, 1973.

10) Branch, H., and G. Moss, Effects on nicotine on rats (albino), Trans. Kansas Acad. Science 41: 317-329, 1938.

11) Buel, F., W. Buel, F. Fielding, D. Little, M. Little, and C. Thienes, Estrus and reproduction in the white rat as influenced by chronic nicotinism, J. Pharmacol. Exp. Ther. 60: 100, 1937.

12) Christie, G. A., Developmental stages in somite and post-somite rat embryos, based on external appearance, and including some features of the macroscopic development of the oral cavity, J. Morpho. 114: 263-281, 1964.

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References

13) De Feo, V. J., Decidualization, in Cellular Biology of the Uterus, ed. R. M. Wynn, Appleton-Century-Crofts, New York, pp. 191-290, 1967.

14) Essenberg, J., J. Schwind, and A. Patras, The effect of nicotine and cigarette smoke on pregnant female albino rats and their offsprings, J. Lab. & Clin. Med., 25: 708-717, 1940.

15) Essenbert, J., L. Fagan, and A. Malerstein, Chronic poisoning of the ovaries and testes of albino rats and mice by nicotine and cigarette smoke, West. J. of Surg. O. & G. 59: 27-32, 1951.

16) Fabro, S., and S. Sieber, Caffeine and nicotine penetrate the pre-implantation blastocyst, Nature 223: 410-411, 1969.

17) Hudson, D. B., and P. S. Timiras, Nicotine injection during gestation: Impairment of reproduction, fetal viability and develepemnt, Biol. of Reprod. 7: 247-253, 1972.

18) King, J. E., and R. F. Becker, Studies on nicotine absorption during pregnancy, I. LD_{50} for pregnant and nonpregnant rats, Am. J. Obst. & Gynec. 95: 508-514, 1966.

19) Larson, P., H. Haag, and H. Silvette, Tobacco-Experimental and Clinical Studies: A Comprehensive Account of the World Literature, Baltimore, Williams & Wilkins Company, 1961.

20) Larson, P. and H. Silvette, Tobacco-Experimental and Clinical Studies: A Comprehensive Account of the World Literature, Baltimore, Williams & Wilkins Company, 1968. Supplement I.

21) Larson, P., and H. Silvette, Tobacco-Experimental and Clinical Studies: A Comprehensive Account of the World Literature, Supplement II, The Williams & Wilkins Company, Baltimore, 1971.

22) Lowe, C. R., Effects of mother's smoking habits on birth weight of their children, Brit. Med. J. 2: 673-676, 1969.

23) McNamara, J. A., Jr., and M. C. McBride, Procion dyes as vital markers in monkeys, J. Dental Res. (in press).

24) Mitchell, J. A., and J. M. Yochim, Influence of environmental lighting on duration of pregnancy in the rat, Endocrinology 87: 472-480, 1970.

25) Mosier, H. D., Jr., and M. Armstrong, Effects of maternal intake of nicotine on fetal and newborn rats, Proc. Soc. Exp. Biol. Med. 116: 956-958, 1964.

26) Noyes, R., and Z. Dickmann, Relationship of ovular age to endometrial developemnt, J. Reprod. and Fertility I: 186-196, 1960.

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27) Orsini, M. W., Technique of preparation, study and photography of benzyl-benzoate cleared material for embryological studies, J. Reprod. Fertil. 3: 283-287, 1961.

28) Orsini, M. W., Study of ovo-implantation in the hamster, rat, mouse, guinea pig and rabbit in cleared uterine tracts, J. Reprod. Fertil. 3: 288-293, 1961.

29) Reynolds, S. R. M., Physiology of the Uterus, Hafner Publishing Co., New York, 1965, p. 529.

30) Schlough, J. S., Delayed implantation in the rat induced by atropine, Biol. Reprod. 1: 315-319, 1969.

31) Seiton, E. C., and M. B. Engel, Reactive dyes as vital indicators of bone growth, Am. J. Anat. 126: 373-392, 1969.

32) Thienes, C. H., Chronic nicotine poisoning, Ann. N.Y. Acad. Sci. 90: 339-348, 1960.

33) Thienes, C., C. Lombard, and A. Leser, Effects of chronic nicotine poisoning of rats on viability of the young and on the adrenal gland, J. Pharmacol. & Exp. Ther. 72: 40-41, 1941.

34) Thienes, C., C. Lombard, F. F. Fielding, A. Lesser, and M. Ellenhorn, Alterations in reproductive functions of white rats associated with daily exposure to nicotine, J. Pharmacol. Exp. Ther. 87: 1-10, 1946.

35) Underwood, P., L. Hester, T. Laffitte Jr., and K. Gregg, The relationship of smoking to the outcome of pregnancy, Am. J. Obst. & Gynec. 91: 270-276, 1965.

36) Wolff, W. A., M. Hawkins, and W. Giles, Nicotine in blood in relation to smoking, J. Pharmacol. Exp. Ther., 95: 145-148, 1949.

37) Yochim, J. M., and V. J. De Feo, Control of decidual growth in the rat by steroid hormones of the ovary, Endocrinology 71: 134-142, 1962.

38) Yochim, J. M., and V. J. De Feo, Hormonal control of the onset, magnitude and duration of uterine sensitivity in the rat by steroid hormones of the ovary, Endocrinology 72: 317-326, 1963.

39) Yochim, J. M., and J. A. Mitchell, Intrauterine oxygen tension in the rat during progestation: Its possible relation to carbohydrate metabolism and the regulation of nidation, Endocrinology 83: 700-713, 1968.

40) Yochim, J. M., and J. A. Mitchell, Influence of environmental lighting on decidualization in the rat, Endocrinology, 87: 465-471, 1970.

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#869A - RUBIN

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

January 16, 1975

Grant Application No. 869A

PHARMACOLOGY

To: The Committee comprising Drs. Feldman, Meier and Sommers

Subject: Ronald P. Rubin, Ph.D., Medical College of Virginia, Richmond, Va.
Continuation application No. 869A
"The Action of Nicotine on the Adrenal Gland"

History:

Grant No. 869 (for calendar 1973) was awarded in the amount of $21,300, with the recommendation for priority in competition for two additional years.

Grant No. 869R1 (first renewal) in the amount of $16,675 was awarded for the period July 1, 1974 through June 30, 1975, without assurance of further support.

Beginning July 1, 1974 Dr. Rubin transferred his research activities from the Downstate Medical Center, New York, to his present location.

Request:

Application No. 869A requests $18,975.

Documents Submitted (attached)

1. Application dated January 2, 1975 (8 pages)
2. Summary Progress Report (5 pages)
3. C.V. of Dr. Rubin (5 pages).
4. Manuscript (in press)
5, Reprint

Comment:

Application No. 869A (as a continuation of the previous two year support by CTR) will compete without priority.

D.S.

DS:wg
Encl.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Grant #869

Application for Research Grant
(Use extra pages as needed)

Date: 1/2/75

1. Principal Investigator (give title and degrees):
Ronald P. Rubin, Ph.D., Professor of Pharmacology and Chief, Autonomic-Cardiovascular Division

2. Institution & address:
Virginia Commonwealth University
Medical College of Virginia
12th & Clay Streets
Richmond, Virginia 23298

3. Department(s) where research will be done or collaboration provided:
Department of Pharmacology

4. Short title of study:
The Action of Nicotine on the Adrenal Gland

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: One to two years

7. Brief description of specific research aims:
Our previous investigations on this project have shown that nicotine enhances basal corticosteroid release and potentiates the steroidogenic effects of adrenocorticotropin (ACTH) on isolated trypsin dispersed cat adrenocortical cells. The details concerning the intimate mechanisms involved in nicotine's action as a secretogogue are still incompletely understood. Insight into the nature of the factors governing its stimulatory effect on the adrenal cortex is of critical importance in gaining a clearer understanding of the molecular processes underlying the fundamental process of secretion. Moreover, knowledge of the effects of nicotine on the gland which plays such significance and importance in light of the widespread use and diverse pharmacologic effects of this alkaloid.

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Thus, the primary aim of this ongoing investigation is to ascertain the nature of the facilitatory action of nicotine on adrenocortical secretion, through a comprehensive study of the relative roles played by calcium, cyclic AMP, and prostaglandins. It is our feeling that the results of this project may lead to a better understanding of some of the health-related problems associated with tobacco smoke. A better understanding of these problems could lead to a more rational approach to their solution.

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8. Brief statement of working hypothesis:

Calcium, cyclic AMP, and prostaglandins have all been implicated in the sequence of events leading to corticosteroid synthesis and release. Therefore, a key to understanding the steroidogenic action of nicotine involves a comprehensive investigation of the possible role(s) played by these putative mediators.

9. Details of experimental design and procedures (append extra pages as necessary)

Structure-Activity Studies. In our continuing investigation on the mechanism of nicotine action as a secretogogue, we recently developed the technique for isolating cat adrenocortical cells by tissue dispersion with trypsin, which enables us to study responses of homogeneous populations of intact cortical cells (see accompanying manuscript by Rubin and Warner for details). The ability of nicotine to augment steroidogenesis in this cortical cell preparation raises the question as to the specificity of this effect. Therefore, experiments are planned to compare the steroidogenic activity of metabolites of nicotine, such as nornicotine and cotinine, as well as related compounds such as lobeline. Such studies will ascertain whether the ability of nicotine to enhance steroidogenesis can be related to its well-defined pharmacologic activity in other biological systems.

Cyclic Nucleotide Studies. Putative mediators of hormone and drug action such as calcium, cyclic AMP, and prostaglandins have been the focus of intensive scientific inquiry over the past several years. Since our previous investigations on this isolated cortical cell preparation have demonstrated that the steroidogenic activity of nicotine is obtunded by calcium deprivation and enhanced by dibutyryl cyclic AMP and by prostaglandin (PGE_2) (Rubin and Warner), follow-up studies are indicated to ascertain whether the action of nicotine involves alterations in the cellular levels of cyclic AMP or prostaglandin.

Preliminary experiments indicate that steroidogenic concentrations of nicotine, like those of ACTH, enhance cyclic AMP levels in isolated cortical cell preparations. It is not clear what relationship, if any, this increase in cyclic AMP has to the process by which nicotine evokes steroid release, since other steroidogenic agents such as the calcium

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9. Details of experimental design and procedures (continued)

ionophore, A-23187, and PGE_2 are unable to significantly elevate cyclic AMP levels, at least in the concentrations so far tested. A more comprehensive investigation -- including exposure to various concentrations of these stimulating agents for different time intervals -- is required before more definitive conclusions concerning the role of cyclic AMP in the steroidogenic activity of nicotine, and other substances, can be derived.

Cyclic GMP is another cyclic nucleotide which is ubiquitously distributed in biological systems and is thought to exert effects which are counter to those of cyclic AMP (Goldberg et al., 1973). This idea of biological "dualism" implies that a cellular event which opposes that produced by an increase in cyclic AMP might be produced by an elevation of tissue cyclic GMP rather than by a "passive" decrease in cyclic AMP. It is therefore of interest to study the effects of nicotine on cortical levels of cyclic GMP. Isolated cat adrenocortical cells will be exposed to graded nicotine concentrations for various time intervals (up to four hours). The cells will be centrifuged and the pellet extracted with trichloroacetic acid. Following centrifugation the supernatant is applied to a AG1X8 column (Bio-Rad) and eluted with perchloric acid to separate cyclic GMP and cyclic AMP. The samples are then lyophilized, diluted with buffer, and assayed for either cyclic AMP or GMP by radioimmunoassay. This technique employs antibodies generated against succinyl-cyclic AMP or succinyl-cyclic GMP (Steiner et al., 1969).

Prostaglandin Studies. Prostaglandins are a group of unsaturated fatty acids which are ubiquitously distributed in tissues and are thought to play a role in endocrine secretory mechanisms (Flack, 1973). Experiments of similar design will be employed to study the effects of nicotine on prostaglandin levels in adrenocortical cells. Antibodies to $PGF_{1\alpha}$ and $PGF_{2\alpha}$ have been made in our laboratory by injecting rabbits with $PGF_{1\alpha}$ or $PGF_{2\alpha}$ which are covalently linked to serum albumin by reaction with carbodiimide reagent. The antisera obtained from these rabbits have been successfully used for determination of tissue $PGF_{1\alpha}$ and $PGF_{2\alpha}$ by radioimmunoassay. Thus, prostaglandin determinations of adrenocortical cells exposed to various nicotine and ACTH concentrations for different time intervals will be carried out. Separation of the various PG's on silicic acid columns precedes the radioimmunoassay procedure. Just as with the cyclic nucleotide studies, an attempt will be made, if possible, to establish a correlation

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9. Details of experimental design and procedures (continued)

between prostaglandin levels and the steroidogenic response. Theoretically, such a correlation could be made either quantitatively and/or temporally.

Calcium Studies. Calcium is another potential mediator of steroidogenesis, since strong evidence exists to support the concept that this action is a necessary factor in ACTH- and nicotine-induced steroidogenesis (see accompanying manuscript, Rubin and Warner). The critical role of calcium may be related to the fact that it, in some as yet unknown manner, permits specific alterations in levels of cyclic nucleotide or prostaglandin which follow stimulation by nicotine. Therefore, the effects of nicotine on cyclic nucleotide and prostaglandin levels will be studied in cortical cells incubated in calcium-deprived media, as well as in calcium-containing media. Experiments are also planned to study the effects of ACTH and nicotine on radiolabeled (^{45}Ca) fluxes, to ascertain whether the actions of these agents are associated with a cellular redistribution of calcium. A characterization of the relationships between calcium, cyclic nucleotides and prostaglandins should contribute greatly to our understanding of the fundamental mechanism whereby nicotine stimulates hormone secretion.

Studies with Radioactive Nicotine. In our soon-to-be published article in the British Journal of Pharmacology, the question was raised as to whether nicotine stimulates steroidogenesis by an action on the surface of cortical cell membrane or whether it must penetrate the cell to elicit its effects. Such information is, of course, critical for ascertaining nicotine's mode of action. Thus, the uptake and accumulation of radioactive (^{3}H or ^{14}C) nicotine in isolated cat adrenocortical cells will be studied over various time intervals, in order to determine whether a relationship exists between nicotine entry into cortical cells and its steroidogenic activity. Cells will be exposed to labeled nicotine for various time intervals and measurement of the radioactive nicotine space carried out. This space is defined as: cpm/gm tissue:cpm/ml of medium. Alternatively, after exposure to nicotine, the cells will be returned to non-radioactive medium and washout of the labeled alkaloid instituted. From derived efflux curves an estimation can be made of the cellular distribution of nicotine. A significant accumulation of nicotine in cortical cells would necessitate additional chemical studies to ascertain whether the alkaloid was present as unchanged nicotine or as its major metabolite, cotinine.

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9. Details of experimental design and procedures (continued)

The general significance of this proposed investigation lies in the fact that the physiological actions of adrenocortical hormones are observed in most tissues and are vital for the normal functioning of many organs and for their responses to stress. In addition, metabolic aberrations which appear during pregnancy, such as toxemia and the diabetogenic state may be related to the increased adrenal function which accompanies gestation. Thus, an agent such as nicotine which can affect steroid hormone production and release will have marked effects on a variety of bodily functions. For example, since adrenal steroids are potent immunosuppressives, the stimulatory effects of nicotine on the cortex may be responsible for the impaired immunological response observed in chronic cigarette smokers (Thomas et al., 1974). Accordingly, in light of the widespread use of nicotine, it is of considerable interest and importance to have a basic understanding of the mechanism whereby this alkaloid elevates steroid levels.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The principal investigator, as Professor of Pharmacology and head of the Cardiovascular and Autonomic Division, has available approximately 2400 square feet of laboratory and office space. The laboratories are well-equipped and house both beta and gamma counting instruments, tissue incubators, automatic pipeting devices, and refrigerated centrifuges. In addition, the principal investigator has access to a well-equipped animal facility run directly by the Department of Pharmacology.

The Thompson-McCaw library, directly across the street from the Pharmacology Department, contains a complete collection of books and journals. The library has direct access to the SUNY and N.L.M. computer search services.

MCV has full computer center facilities and these services are directly available to the project. The investigator's laboratory also has a Hewlett-Packard (Model 9810) programmable calculator for more routine calculations.

The Department has a small shop and a trained mechanic,and the Medical College has fully-equipped mechanical, instrument and electronic shops which are available through the Department of Biophysics.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

see enclosed curriculum vitae.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

see enclosed curriculum vitae.

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R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Ronald P. Rubin (Principal Investigator)	40	---
Technical:		
Research Assistant	100	REDACTED
*includes 11% for fringe benefits		
		REDACTED
Sub-Total for A		

B. Consumable supplies (by major categories)

Glassware	$ 1,000
Radioactive Chemicals	1,000
Animals	1,000
Other Miscellaneous Lab Items	1,000
Sub-Total for B	$ 4,000

C. Other expenses (itemize)

Travel (to attend scientific meetings)	
Sub-Total for C	$ 500
Running Total of A + B + C	R

D. Permanent equipment (itemize)

Sub-Total for D	-0-
E. Indirect costs (15% of A+B+C)	2,475
Total request	$18,975

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	4,000	500	--	2,475	18,975
Year 3		4,000	500	--	2,475	18,975

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R: REDACTED MATERIAL

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Action of Calcium on Secretion	AM 18066 PHRA	52,407	6/1/74 - 2/28/77

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Ronald P. Rubin, Ph.D.

Signature Ronald P. Rubin Date 1/5/75

Telephone R
Area Code Number Extension

Check's payable to:
Mr. Raymond Holmes, Jr.
Vice President for Finance
Virginia Commonwealth University
Mailing address for checks

1200 East Broad Street

Richmond, Virginia 23298

Responsible officer of institution

Typed Name Dr. John J. Salley

Title Assoc. Vice Pres for Research & Graduate Affairs

Signature John J. Salley Date 1/9/75

Telephone R

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Summary Progress Report

CTR Grant #869	Progress Report #2 6/1/74 - 1/1/75
Name of Investigator:	Ronald P. Rubin, Ph.D.
Name of Institution:	Virginia Commonwealth University Medical College of Virginia
Mailing Address:	Department of Pharmacology (Box 726) Medical College of Virginia Richmond, Virginia 23298
Title of Grant:	The Action of Nicotine on the Adrenal Gland

During the past year and a half our laboratory has been engaged in a comprehensive study of the stimulatory effects of nicotine on the cat adrenal gland. The major thrust of this investigation has been from two major perspectives: (a) to elucidate the role of cyclic AMP in the mechanism whereby nicotine, acetylcholine and other medullary secretogogues enhance catecholamine secretion and (b) to discern whether nicotine exerts a direct action on adrenocortical cells, and if so, by what mechanism. Since the details of this work are elaborated and conclusions amply documented in the accompanying reprint (Jaanus and Rubin, 1974) and preprint (Rubin and Warner, in press), only the highlights of these studies will be presented here.

(a) Medulla. Earlier work established that calcium is an absolute requirement for nicotine and acetylcholine-induced release of catecholamines by the cat adrenal medulla (Douglas and Rubin, 1961b). Since cyclic AMP has been implicated as a "second messenger" in many physiological and pharmacological responses (Robison et al., 1971), it is well understood that the elucidation of the nature of calcium action in nicotine-induced catecholamine release requires intimate knowledge of the role of cyclic AMP. Therefore, in collaboration with Dr. Siret Jaanus (Jaanus and Rubin, 1974), the effects of nicotine on cyclic AMP levels and catecholamine release were determined in cat adrenal glands perfused in situ with Locke's solution.

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Initially, cyclic AMP analyses (method of Steiner et al., 1969) were carried out on the cat medulla and cortex, after separating these two organs. The mean basal levels in medulla and cortex were 30 ± 13 and 172 ± 6 pmoles/gland, respectively. Thus, establishing the presence of cyclic AMP in the medulla, experiments were carried out to discern if a quantitative and/or temporal correlation existed between medullary cyclic AMP levels and catecholamine secretion following stimulation by nicotine. Indeed, perfusion with nicotine or acetylcholine (4×10^{-5} g/ml) elevated adrenal cyclic AMP levels and enhanced its rate of release into the adrenal perfusate. However, the time course of the changes in tissue cyclic AMP during stimulation was out of phase with the time course of catecholamine release. Maximal increases in cyclic AMP were not manifest until after eight minutes of exposure to the secretogogue, whereas maximal rates of secretion occurred during the first minute. The lack of correlation between adrenal cyclic AMP levels and catecholamine release was also reflected in experiments with theophylline, an inhibitor of phosphodiesterase. Theophylline, in concentrations which augment adrenal cyclic AMP levels, failed to enhance basal catecholamine release and did not potentiate the secretory response to a submaximal concentration of nicotine.

These data are interpreted to mean that the elevation of secretion effected by nicotine is not directly monitored by adrenal cyclic AMP levels in a manner which resembles that of calcium. These findings do not mitigate the possibility, however, that one or more alternate mediators are interposed between the response triggered by nicotine and the release of hormone. Therefore, experiments are now in progress to determine whether the stimulatory action of nicotine on the adrenal medulla is better correlated with alterations in cyclic GMP and/or prostaglandin levels.

Cortex. Up to now, controversy has existed as to whether nicotine is able to elevate corticosteroid secretion by a direct action on adrenocortical cells (Kershbaum et al., 1968; Suzuki et al., 1973). Our recent work has clearly established (see preprint) that nicotine possesses the potentiality of directly stimulating isolated cat adrenocortical cells. The stimulant action manifests itself in micromolar concentrations, is dose-dependent and like the physiological stimulus, ACTH, depends upon the presence of calcium. Not only is nicotine able to stimulate cortical cells directly but it enhances the steroidogenic effect of ACTH in an additive, rather than synergistic, manner.

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The further observations that nicotine enhances the steroidogenic action of exogenous cyclic nucleotide and prostaglandin E_2, just as it enhances the action of ACTH, suggest that these proposed mediators of steroidogenesis play crucial roles in mediating the action of nicotine. However, support of such an hypothesis requires the measurement of changes in tissue levels of these putative mediators during stimulation by nicotine, and these experiments are now in progress.

The role of calcium in the steroidogenic action of nicotine also cannot be defined at present. Nicotine-like acetylcholine stimulates the medulla to secrete by depolarizing the chromaffin cell membrane (Douglas et al., 1967), facilitating transmembrane calcium flux, which in turn triggers the release of preformed catecholamine stored in secretory granules (see Rubin, 1970). On the other hand, in the cortex there appears to be no correlation between steroidogenic activity and the depolarization of cortical cells (Jaanus et al., 1970), and stimulation by ACTH results from an intracellular translocation of calcium (Jaanus and Rubin, 1971). Since nicotine can traverse cell membranes and release calcium from cellular binding sites (Weiss, 1968) an intracellular mobilization of calcium may be responsible for nicotine-induced steroidogenesis. Experiments are now in progress to compare the effects of ACTH and nicotine on radiocalcium fluxes in the isolated cat adrenocortical preparation. Such studies will provide salient information as to whether the action of nicotine on the adrenal cortex is primarily associated with a transmembrane flux of extracellular calcium or a redistribution of intracellular calcium.

Although our studies have uncovered valuable information concerning the mode of action of nicotine on adrenal hormone release, we still have an incomplete picture of the processes involved in this action. Despite the recognized importance of nicotine, as a result of its widespread use, there have been surprisingly few experimental studies concerned with the mechanism by which this pharmacologic agent affects non-neuronal endocrine cells. There is therefore an obvious gap in our knowledge regarding cellular events occurring during nicotine-induced activation of the secretory process. Advances made in our knowledge of the intimate mechanism of nicotine action by a continuation of this study would have important implications and immense value in regard to understanding and treating the pathophysiological states related to the chronic use of tobacco.

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References alluded to in the sections on experimental design and in the Summary Progress Report

Carchman, R.A., Jaanus, S.D. and Rubin, R.P. Mol. Pharmacol. 7: 491 (1971).

Douglas, W.W., Kanno, T. and Sampson, S. J. Physiol. (London) 188: 107 (1967).

Douglas, W.W. and Rubin, R.P. J. Physiol. (London) 159: 40 (1961a).

Douglas, W.W. and Rubin, R.P. Nature (London) 192: 1087 (1961b).

Flack, J.D. I. The Prostaglandins (Volume I), P.W. Ramwell, ed., pg. 327, Plenum Press (New York, 1973).

Jaanus, S.D., Rosenstein, M.J. and Rubin, R.P. J. Physiol. (London) 209: 539 (1970).

Jaanus, S.D. and Rubin, R.P. J. Physiol. (London) 213: 581 (1971).

Jaanus, S.D. and Rubin, R.P. J. Physiol. (London) 237: 465 (1974).

Goldberg, N.D., O'Dea, R.F., Haddox, M.K. In. Adv. in Cyclic Nucleotide Res. 3: 155 (1973).

Kershbaum, A., Pappajohn, D.J., Bellet, S., Hirabayashi, M. and Shafiiha, H. J. Amer. Med. Assn. 203: 275 (1968).

Robison, G.A., Butcher, R.W. and Sutherland, E.W. In: Cyclic AMP: Academic Press (New York, 1971).

Rubin, R.P. Pharmacol. Rev. 22: 389 (1970).

Rubin, R.P., Jaanus, S.D. and Carchman, R.A. Nature (London) 240: 150 (1972).

Rubin, R.P. and Warner, W. Brit. J. Pharmacol. (in press) (1975).

Steiner, A.L., Kipnis, D.M., Utiger, R. and Parker, C. Proc. Nat. Acad. Sci. 64: 367 (1969).

Suzuki, T., Ikeda, H. and Narita, S. Quart. J. Exp. Physiol. 58: 139 (1973).

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Thomas, W.R., Holt, P.G., Keast, D. Nature (London) 248: [illegible] in the 358 (1974). Cross Readit

Weiss, G.B. J. Pharmacol. Exp. Ther. 163: 43 (1968). [illegible]

Douglas, W.W., Kanno, T. and Sampson, S. J. Physiol. (London) 188: 107 (1967).

Douglas, W.W. and Rubin, R.P. J. Physiol. (London) 159: [illegible]

Douglas, W.W. and Rubin, R.P. Nature (London) 192: 1087 (1967).

[illegible]

Jaanus, S.D., Rosenstein, M.J. and Rubin, R.P. J. Physiol. (London) [illegible]

[illegible]

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Shafiiha, H. J. Amer. Med. Assn. 203: 275 (1968).

[illegible]

Rubin, R.P. [illegible]

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Thomas, W.R., Holt, P.G., Keast, D. Nature (London) 248: 358 (1974).

Weiss, G.E. J. Pharmacol. Exp. Ther. 184: 47 (1968).

CURRICULUM VITAE
Ronald P Rubin

Date & Place of Birth: REDACTED

Marital Status: REDACTED

Education:

Harvard University	A.B. (Biochemistry)	1954
Attended Harvard School of Dental Medicine		1954-57
Harvard Graduate School of Education	A.M.T. (Science Ed.)	1958
Albert Einstein College of Medicine	Ph.D. (Pharmacology)	1963

Positions:

1974-present	Professor & Chief, Autonomic-Cardiovascular Division, Department of Pharmacology; Medical College of Virginia
1970-1974	Associate Professor, Department of Pharmacology; State University of New York, Downstate Medical Center
1966-1970	Assistant Professor, Department of Pharmacology; State University of New York; Downstate Medical Center
1964-1966	Instructor, Department of Pharmacology; State University of New York; Downstate Medical Center
February 1964-June 1964	Instructor, Department of Physiology; Hunter College; New York City (Evenings)
1963-1964	Postdoctoral Fellow, Department of Pharmacology; Albert Einstein College of Medicine
1958-1959	Science Instructor, White Plains High School; White Plains, New York

Society Memberships:

REDACTED

Committee Memberships:

Committee of the Faculty, 1972; Medical School Admissions Committee, 1972 (Downstate Medical Center); FASEB Public Information Committee for ASPET (1972-1975)

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

BIBLIOGRAPHY

Publications

1. Douglas, W.W. and Rubin, R.P. Mechanism of nicotinic action at the adrenal medulla: calcium as a link in stimulus-secretion coupling. Nature 192: 1087-1089 (1961).

2. Douglas, W.W. and Rubin, R.P. The role of calcium in the secretory response of the adrenal medulla to acetylcholine. J. Physiol. 159: 40-57 (1961).

3. Douglas, W.W. and Rubin, R.P. The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. J. Physiol. 167: 288-310 (1963).

4. Douglas, W.W. and Rubin, R.P. The stimulant action of barium on the adrenal medulla. Nature 203: 305-307 (1964).

5. Douglas, W.W. and Rubin, R.P. The effect of alkali earths and other divalent cations on adrenal medullary secretion. J. Physiol. 175: 231-241 (1965).

6. Douglas, W.W., Poisner, A.M. and Rubin, R.P. Efflux of adenine nucleotides from perfused adrenal glands exposed to nicotine and other chromaffin cells stimulants. J. Physiol. 179: 130-137 (1966).

7. Rubin, R.P. and Jaanus, S.D. A study of the release of catecholamines from the adrenal medulla by indirectly acting sympathomimetic amines. Arch. Exp. Path. Pharmak. 254: 125-137 (1966).

8. Rubin, R.P. and Jaanus, S.D. The release of nucleotide from the adrenal medulla by indirectly acting sympathomimetic amines. Biochem. Pharmacol. 16: 1007-1012 (1967).

9. Rubin, R.P. and Jaanus, S.D. Tachyphylaxis to the stimulant actions of the indirectly acting sympathomimetic amines and acetylcholine on the adrenal medulla. Arch. Exp. Path. Pharmak. 256: 464-475 (1967).

10. Rubin, R.P., Feinstein, M.B., Jaanus, S.D. and Paimre, M. Inhibition of catecholamine secretion and calcium exchange in perfused cat adrenal glands by tetracaine and magnesium. J. Pharmac. Exp. Ther. 155: 463-471 (1967).

11. Jaanus, S.D., Miele, E., and Rubin, R.P. The analysis of the inhibitory effect of local anesthetics and propranolol on adreno-medullary secretion evoked by calcium or acetylcholine. Brit. J. of Pharmac. & Chemother. 31: 319-330 (1967).

12. Rubin, R.P., Miele, E. and Jaanus, S.D. Inhibition of adreno-medullary secretion by local anesthetics and proprandolol. The Pharmacologist 9 (1967).

1003546489

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

13. Miele, E. and Rubin, R.P. Further evidence for the dual action of local anesthetics on the adrenal medulla. J. Pharmac. Exp. Therap. 161: 296-301 (1968).

Publications

14. Jaanus, S.D., Miele, E. and Rubin, R.P. The action of guanethidine on the adrenal medulla of the cat. Brit. J. Pharmac. Chemother. 33: 560-569 (1968).

15. Rubin, R.P. and Miele, E. The relation between the chemical structure of local anesthetics and inhibition of calcium-evoked secretion from the adrenal medulla. Arch. Exp. Path. Pharmak. 260: 298-308 (1968).

16. Rubin, R.P., Cohen, M.S., Harman, S.M. and Roer, E.M. The localization of adrenaline-rich medullary chromaffin cells adjacent to the adrenal cortex. J. Endocr. 41: 541-545 (1968).

17. Rubin, R.P. and Miele, E. A study of the differential secretion of epinephrine and norepinephrine from the perfused cat adrenal gland. J. Pharmac. Exp. Therp. 164: 115-121 (1968).

18. Rubin, R.P. The metabolic requirements for catecholamine release from the adrenal medulla. J. Physiol. 202: 197-200 (1969).

19. Rubin, R.P. The role of energy metabolism in calcium-evoked secretion from the adrenal medulla. J. Physiol. 206: 181-192 (1970).

20. Rubin, R.P., Jaanus, S.D. and Miele, E. Calcium-dependent corticosteroid release from the perfused cat adrenal gland. Experientia (Basel) 25: 1321-1323 (1969).

21. Jaanus, S.D., Rosenstein, M.J. and Rubin, R.P. On the mode of action of ACTH on the isolated perfused adrenal gland. J. Physiol. 209: 539-556 (1970).

22. Jaanus, S.D. and Rubin, R.P. The effect of ACTH on calcium distribution in the perfused cat adrenal gland. J. Physiol. 213: 581-598 (1971).

23. Carchman, R.A., Jaanus, S.D. and Rubin, R.P. The role of adrenocorticotropin and calcium in adenosine cyclic 3'-5' phosphate production and steroid release from the isolated, perfused cat adrenal gland. Molec. Pharmacol. 7: 491-499 (1971).

24. Rubin, R.P., Carchman, R.A. and Jaanus, S.D. Role of cyclic 3'5' adenosine monophosphate on corticosteroid synthesis and release from the intact gland. Biochem. Biophys. Res. Commun. 47: 1492-1497 (1972).

25. Jaanus, S.D., Carchman, R.A. and Rubin, R.P. Further studies on the relationship between cyclic AMP levels and adrenocortical activity. Endocrinology 91: 887-895 (1972).

26. Rubin, R.P., Jaanus, S.D. and Carchman, R.A. The role of calcium and adenosine cyclic 3'5' phosphate in the action of adrenocorticotropin. Nature 240: 150-152 (1972).

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

27. Rubin, R.P., Jaanus, S.D., Carchman, R.A. and Puig, M. Reversible inhibition of ACTH-induced corticosteroid release by cycloheximide: Evidence for an unidentified cellular messenger. Endocrinology 93: 575-580 (1973).

28. Jaanus, S.D. and Rubin, R.P. Analysis of the role of cyclic adenosine 3'5' monophosphate in catecholamine release. J. Physiol. 237: 465-476 (1974).

29. Rubin, R.P., Sheid, B., McCauley, R., Laychock, S.G. ACTH-induced protein release from the perfused cat adrenal gland: Evidence for exocytosis? Endocrinology 95: 370-378 (1974).

30. Laychock, S.G. and Rubin, R.P. Isolation of ACTH-induced protein from adrenal perfusate. Steroids 24: 177-184 (1974).

31. Rubin, R.P. and Warner, W. Nicotine-induced stimulation of steroidogenesis in adrenocortical cells of the cat. Brit. J. Pharmacol.

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Reviews, Monographs, and Book Chapters

1. Rubin, R.P. The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol. Rev. 22: 389-428 (1970).

2. Jaanus, S.D. and Rubin, R.P. The perfused adrenal gland: In Hormones and Cyclic Nucleotides (a volume of Methods in Enzymology): Submitted for publication.

3. Rubin, R.P. Calcium and the Secretory Process. Plenum Press, New York (1974).

4. Rubin, R.P. The role of calcium in drug action. Presented at the International Symposium on Drug Interactions. Milan, Italy: September 17-19, 1973. In Drug Interactions, P.L. Morselli, S. Garattini and S.N. Cohen, editors, Raven Press (New York), pp. 163-172 (1974).

1003546492

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Selected Abstracts and Local Citations

1. Douglas, W.W. and Rubin, R.P. The mode of action of acetylcholine son the adrenal medulla. Proc. 1st Int. Pharmacol. Meet., Stockholm, Sweden, Biochem. Pharmacol. 8: 20 (1961).

2. Douglas, W.W. and Rubin, R.P. The role of calcium in adrenal medullary secretion evoked by acetylcholine or potassium. J. Physiol. 159: 24-25P (1961).

3. Douglas, W.W., Poisner, A.M. and Rubin, R.P. Stimulus-secretion coupling at the adrenal medulla. XXII Int. Cong. of Physiol. Sciences, Leiden, Excerpta Medica, Int. Cong. Series 48: 827 (1962).

4. Rubin, R.P. and Douglas, W.W. The alkali earths and catecholamine release. Fed. Proc. 22 (2): 508 (1963).

5. Jaanus, S.D. and Rubin, R.P. The action of the sympathomimetic amines on the adrenal medulla. The Pharmacologist 7: 168 (1965).

6. Rubin, R.P., Jaanus, S.D., Feinstein, M.B. and Paimre, M. Inhibition of calcium evoked secretion of adrenal medulla by local anesthetics. The Pharmacologist 7: 169 (1965).

7. Rubin, R.P. and Jaanus, S.D. The release of nucleotide from the adrenal medulla by sympathomimetic amines. The Pharmacologist 8: 194 (1966).

8. Miele, E. and Rubin, R.P. Secretion of adrenaline and noradrenaline from the perfused cat adrenal gland. Proc. of the Joint Meet. Between the Italian and Brit. Pharmacological Societies; September 12-13, 1968; Florence, Italy. In Brit. J. Pharmacol. 34: 691P (1968).

9. Carchman, R.A., Jaanus, S.D. and Rubin, R.P. Action of ACTH and calcium on adrenal cyclic AMP production and steroid release. Fed. Proc. 30 (2): 307 (1971).

10. Rubin, R.P., Carchman, R.A., Jaanus, S.D. and Feinberg, M.B. The role of ACTH and cyclic AMP in corticosteroid release from the adrenal cortex. Abstr. Physiology and Pharmacology of Cyclic AMP (In International Conference), Milan, Italy; July 1971; p. 126.

11. Carchman, R.A., Jaanus, S.D. and Rubin, R.P. Effect of divalent cations on adrenal adenyl-cyclase activity. Fed. Proc. 31 (2): 294 (1972).

12. Jaanus, S.D., Carchman, R.A. and Rubin, R.P. Effect of increased cyclic AMP levels on adrenal steroid production and secretion. Abstr. Fifth International Congress on Pharmacology; July 1972; San Francisco, Calif.; p. 113.

13. Warner, W. and Rubin, R.P. Action of ACTH on isolated cat adrenocortical cells. Fed. Proc. 33 (3): 205 (1974).

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

February 3, 1975

Grant Application No. 864B

PHARMACOLOGY

To: The committe comprising Drs. Bing, Gardner and Meier

Subject: Theodore Alan Slotkin, Ph.D., Duke University Medical Center
Durham
"Effects of Nicotine on Sympatho-Adrenal Development"

History

Grant No. 864, for the year 1973, was awarded in the amount requested ($12,110) without assurance of continued support. Continuation support (Grant No. 864A) for this work "Maturation of the Adrenal Medulla: Catecholamine Stores in Normal and Hypertensive Rats" was awarded by the SAB in October 1973, in the amount of $13,346 for the period January 1, 1974 through December 31, 1974.

Request

Application No. 864B requests $18,359 for the first year of a three year program. The title change reflects the new area of emphasis in the present proposal.

Documents Submitted (attached)

1. Application dated January 20, 1975 (21 pages, including CV of Dr. Slotkin).
2. Progress Report No. 4, covering period July 1, 1974 - December 31, 1974 (3 pages).
3. Five reprints (with CTR acknowledgment).
4. Two manuscripts (in press), one with CTR acknowledgment.

D.S.jmb
Enclosures

D.S.

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JAN 31 1975

Application for Research Grant
(Use extra pages as needed)

Date: 1-20-75

1. Principal Investigator (give title and degrees):

Theodore Alan Slotkin, Ph.D.
Assistant Professor of Pharmacology

2. Institution & address:

Duke University Medical Center
Department of Physiology and Pharmacology
Durham, North Carolina 27710

3. Department(s) where research will be done or collaboration provided:

Department of Physiology and Pharmacology

4. Short title of study:

EFFECTS OF NICOTINE ON SYMPATHO-ADRENAL DEVELOPMENT

5. Proposed starting date: 7/1/75

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Many autonomic drugs and drugs which act in the CNS exert similar direct or reflex effects on catecholamine stores in adrenergic neurons and in the adrenal medulla of the mature organism; however, the actions of these drugs are often markedly different (and even opposite) in fetal or neonatal animals. The proposed studies will determine how maturational changes in the rat adrenal medulla alter the effects of nicotine on that tissue. In addition, since the maturation of the adrenal medulla is itself dependent upon biochemical and neuronal feedback mechanisms, this study will also examine how pre- and post-natal exposure to nicotine alters the subsequent maturation of normal sympatho-adrenal function.

In the time period outlined, we expect to determine:

(a) The acute and chronic effects of nicotine on the mature adrenal medulla (already in progress).

(b) The effects of acute and chronic nicotine on post-natal adrenergic development.

(c) The long-term effects of pre-natal exposure to nicotine on the developing adrenal medulla.

(d) Whether pretreatment with nicotine blocking agents alters developmental effects of nicotine.

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8. Brief statement of working hypothesis:

The adrenergic nervous system and adrenal medulla play vital roles in the maintenance of the cardiovascular system, normal metabolic function and in the response to various stress situations. In the neonate, these tissues are markedly immature in their capability to synthesize, store and secrete catecholamines. The deficiencies in the adrenal medulla result in part from age-dependent alterations in the function and contents of storage vesicles, which are in turn dependent upon neural stimulation. These studies will elucidate many of the factors operating in the development of sympathetic amine stores. The rates of synthesis of adrenal catecholamines, storage vesicles, and the development of storage capabilities will be measured as well as the functioning of the adrenal gland in response to neural stimulation and nicotine-induced amine depletion. Data from several studies suggest that there are crucial differences between adults and neonates in amine storage capabilities and in the effects of drugs (such as nicotine) which act either directly on the adrenal or which elicit reflex stimulation of the sympatho-adrenal axis. Using the adrenal medulla as a model, some of these differences in drug action can be elucidated.

Fetuses are often exposed to a wide variety of drugs known to affect catecholamines, either as a result of clinical treatment or maternal self-

(continued on page 6)

9. Details of experimental design and procedures (append extra pages as necessary)

(a) Introduction

1. Previous work done by applicant:

The major effort of the applicant has been directed toward elucidating the mechanisms of regulation on the synthesis, uptake, storage and release of the catecholamines of the adrenal medulla. Besides the inherent interest in studying this tissue, the adrenal medulla is often utilized as a model of the adrenergic neuron; both tissues arise embryonically from the neural crest and both have the ability to synthesize, store and secrete catecholamines. Each contains storage vesicles which can accumulate amines by a mechanism which is stimulated by ATP-Mg^{2+} and blocked by reserpine. The vesicles contain dopamine β-hydroxylase (DBH), chromogranins and adenine nucleotides as well as catecholamines; it is accepted generally that the catecholamine and adenine nucleotides (primarily ATP) form a storage complex in a molar ratio of 4 to 1.

Techniques developed to a large extent in this laboratory enable the evaluation of many of the factors which influence catecholamine disposition from the point of view of alterations in storage vesicles. Initial studies concentrated on the mechanism by which storage vesicles are assembled administration of drugs (insulin, reserpine) which elicit reflex sympatho-adrenal discharge led to the formation of large numbers of new, "immature" vesicles which had properties different from the normal population (Slotkin and Kirshner, 1973a,b; Slotkin and Edwards, 1973):

(1) The new vesicles showed a decreased preference for uptake of epinephrine vs. metaraminol.
(2) There was a decreased buoyant density in the new vesicles.
(3) There was a decreased ratio of catecholamines to ATP in the new vesicles.
(4) The catecholamine content per vesicle was decreased.

Another situation in which large numbers of new vesicles have to be synthesized is in maturation. Studies from other laboratories (Ignarro and Shideman, 1968; Waymire et al., 1974; Daikoku et al., 1969; Elfvin, 1967) indicated that in rats and chicks, catecholamine biosynthetic enzymes appeared early in gestation but catecholamines were not detectable until much later, when storage vesicles could first be detected by electron microscopy (18th day of gestation). Consequently, most of the maturational increases in catecholamines occur postnatally.

(Continued on page 6).

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratory consists of 1300 square feet fitted with standard laboratory type benches. Major items of equipment include: Sorvall RC-2B centrifuge, Beckman L5-50 ultracentrifuge and rotors, Farrand ratio fluorometer, catecholamine autoanalyzer, Wang 600-6-TP programmable calculator, incubation baths, pH meter, balances and other general items of laboratory hardware. Research facilities include animal rooms, cold rooms, a spectrophotometer and a liquid scintillation counter.

11. Additional facilities required:

The current RC-2B is in heavy, continuous use by five people in this laboratory and is quite old. Funds are therefore requested for another centrifuge for the proposed studies.

12. Biographical sketches of investigator(s) and other professional personnel (append):

See pages 17, 18, 19 and 20.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See page 21.

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Theodore A. Slotkin - Principal Investigator	50	0
Technical		
Christopher Lau (B.S.)	80	REDACTED
Fringe Benefits (12.6%)		
Sub-Total for A		R
B. Consumable supplies (by major categories)		
Animals, including housing and shipping costs (100 male rats @ $3.00, 100 pregnant rats @ $10.00, $200 shipping, housing @ 10¢/day for avg. 50 days)		2500
Radioisotopes (^{3}H-epinephrine, metaraminol, tyramine; ^{14}C-tyrosine & epinephrine)		1750
Chemicals and hardware		750
Sub-Total (B)		5000
C. Other expenses (itemize)		
Equipment maintenance and service		500
Travel to ASPET Spring and Fall Meetings		500
Sub-Total for C		1000
Running Total of A + B + C		R
D. Permanent equipment (itemize)		
Sorvall RC-2B centrifuge and rotors		4000
Sub-Total for D		4000
E. Indirect costs (15% of A+B+C)		1873
Total request		$18359

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	5000	1000	0	1931	14807
Year 3		5000	1000	0	1994	15282

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R: REDACTED MATERIAL

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Effects of morphine on the adrenal medulla	DA-00465	$85,000	6/1/73-5/31/76
2. Hypertensive rats	American Heart Assn.	$50,000	10/1/73-9/30/76

These projects are unrelated to the present proposal and provide salary support for the investigator and two post-doctoral fellows and laboratory expenses for these projects.

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Theodore Alan Slotkin

Signature Theodore Slotkin Date 1/20/75

Telephone R
Area Code Number Extension

Checks payable to

Duke University

Mailing address for checks

Vice President, Business & Finance

Duke University

Durham, North Carolina 27706

Responsible officer of institution:

Typed Name W. G. Anlyan, M.D.

Title Vice President for Health Affairs

Signature W G Anlyan Date 1/28/75

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8. (continued from page 2)

administration; although catecholamine stores undergo marked maturational changes, little is known about the effects of prenatal drug exposure on the subsequent developmental process. A number of studies suggest that even single doses of drugs at critical stages in development may alter permanently the disposition of catecholamines in central and peripheral adrenergic tissue. The proposed experiments will identify on the biochemical and functional levels, specific loci of nicotine-induced maturational alterations, and will show whether pretreatment with nicotinic blocking agents can prevent the long-term effects of nicotine. These studies may be of considerable importance in evaluating the consequences of exposure to nicotine during pre- and postnatal development.

9. (continued from page 2)

(a) Introduction

1. Previous work done by applicant (con't.):

Recent studies by the applicant (Slotkin, 1973a,b; 1974a) examined the maturation of the rat adrenal medulla from the point of view of changes in the number and properties of storage vesicles. This first entailed modification of the available methodology to enable measurements utilizing small amounts of tissue; the methods are described in detail in the appended reprints and in the "Methods" section.

At birth, the number of storage vesicles (as determined by DBH, an enzyme marker for vesicles) was quite low, and the vesicles differed markedly from adult vesicles in that they were considerably more dense on sucrose density gradients. The density difference was confirmed by determination of the subcellular distribution of catecholamines - lower levels were found in fractions usually containing "light" vesicles. Other properties of the vesicles were found to be different from adults neonatal vesicles were more fragile and displayed greater susceptibility to osmotic shock; however, the stability of amine storage was normal as was the ratio of catecholamines to ATP (the putative components of the storage complex). On the functional level, the uptake of amines into the vesicles appeared to be normal, but neonates given insulin did not evidence a normal catecholamine secretory response; however, neonatal adrenals excised and incubated in high-potassium Locke's solution did secrete normally, indicating that the lack of secretion in vivo is probably due to non-functional innervation of the gland by the splanchnic nerve.

Sometime between birth and 10 days of age, a complete alteration of sympatho-adrenal function occurs: the secretory response becomes normal, "light" vesicles appear, DBH, ATP and catecholamines increase markedly and the vesicle density shifts such that vesicles are now lighter than in adults. Furthermore, at this stage there is a partial loss of normal preference for uptake of catecholamines vs. non-catecholamines (metaraminol) and the stability of storage is impaired. These are all signs of increased neural input to the gland and the vesicles are similar to those found in stressed adult animals (in which increased neural stimulation also occurs). These qualitative and quantitative alterations in function and properties gradually approach those in normal adults by 40-50 days of age, after which additional altera-

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tions reflect only increases in the number of vesicles.

To summarize these results, the maturation of the adrenal medulla is dependent upon neural input to the gland. At birth, no input occurs, vesicle turnover is low, and dense, overloaded vesicles are present. By 10 days, innervation is functional, turnover increases markedly, and "light" vesicles appear with altered uptake and storage capabilities.

Additional studies have been conducted to examine these changes in terms of kinetic analysis of uptake. The Km for epinephrine (E) remained at about 30 μM throughout development but the maximal uptake (Umax) at birth was 30 nmols/30 min/100 μg endogenous catecholamines compared to 20 nmols at subsequent ages. The Km for metaraminol (MA) was about 4 mM at birth, 0.7 mM at 10-20 days of age and 1.2 mM thereafter, while Umax was approximately 170 nmols initially, 50 nmols at 10-20 days and 70 nmols thereafter. The preference of the vesicles for uptake of E vs. MA (both at 0.1 mM) was approximately 6 to 1 at birth, 1 to 1 at 10 days, 3.5 to 1 at 20 days and 5 to 1 thereafter, while the preference for E vs. tyramine was 2 to 1 except at 10 days, when the ratio was 1 to 1. These data suggest that the two vesicular uptake systems, typified by incorporations of E and of MA, develop independently of each other and that the age-dependent alterations are present both at the level of the vesicle membrane and intravesicular storage. A kinetic model was developed to aid in the identification of probable sites of specific age-dependent alterations. Maturational changes in uptake and specificity produced parallel changes in synthesis of catecholamines as determined by conversion of newly-incorporated tyramine to octopamine. Furthermore, these alterations could affect the actions of adrenergic false transmitters (which displace catecholamines from vesicles) on immature rats.

These data indicate that any study of drug effects on development of catecholamine stores (such as this proposal) must comprise at least three elements:

(1) Evaluation of the effect of the drug in adults;
(2) Evaluation of how the maturational alterations change the action of the drug in the developing organism; and
(3) Evaluation of how drug administration alters subsequent development of catecholamine stores.

One drug which we are presently studying in this fashion is morphine (Anderson and Slotkin, 1974; Slotkin and Anderson, 1974a,b). Morphine was administered twice daily to adult rats and the adrenals were analyzed for catecholamines (CA), tyrosine hydroxylase (TH) activity and dopamine β-hydroxylase (DBH) activity. Twenty-four hours after dosage with 10 mg/kg, CA were reduced and TH and DBH slightly elevated. After one week of treatment, all 3 were elevated. Subsequent dose increments resulted in acute decreases in CA and DBH and increases in TH, followed by dose-dependent increases in CA, DBH and TH to a maximum of 2-3 times control levels after 2 weeks at 100 mg/kg. Withdrawal led to a decline to control levels within 10 days. Shifts in the subcellular distribution of DBH suggested that morphine administration increased the rate of vesicle synthesis, and measurements of the uptakes of ^{14}C-epinephrine and ^{3}H-metaraminol into isolated storage vesicles indicated an increased proportion of "immature" vesicles. Prolonged administration of large doses of morphine led to the formation of vesicles with an apparently defective amine uptake mechanism; these vesicles also displayed abnormally low fragility and a reduced rate of spontaneous CA efflux. These data suggest that (1) tolerance to morphine-induced sympatho-adrenal discharge does not develop and that the recovery from

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the acute CA depletion results from increased CA synthesis and storage, and (2) morphine produces a persistent change in the properties of adrenal medullary storage vesicles.

On the other hand, preliminary studies with perinatally-addicted developing rats demonstrated changes completely different from those seen in adults; catecholamine levels and dopamine β-hydroxylase activity were reduced compared to controls and no induction of tyrosine hydroxylase was observed. The time course of adrenomedullary maturation was delayed through the first 10-20 days of age, with reduced numbers of storage vesicles and larger proportions of partially filled vesicles. On exposure to morphine, continued until weaning, perinatally addicted rats did not display any of the changes in catecholamine synthesis or uptake seen in adult rats, but we have preliminary evidence that defects may develop post-weaning even if morphine is discontinued. The differences between adults and developing rats can be partly explained by the absence of functional innervation of the neonatal adrenal medulla, but the inability of morphine to cause induction after 10 days may indicate a "re-programming" of the relationship between neural input and development of the adrenal medulla.

Additional studies are being conducted with adult and developing spontaneously hypertensive rats (SHR), where a genetic abnormality (hypertension) is associated with alterations in sympatho-adrenal catecholamine disposition (Slotkin and Green, 1974; Slotkin, 1974b). Specific loci of alterations in synthesis, uptake, storage and release have been identified in a fashion analogous to studies with drugs.

Current research efforts are concentrating on the elucidation of the acute and chronic effects of nicotine on the mature rat adrenal. Preliminary results indicate a similarity to the effects of other sympatho-adrenal stimulators, such as morphine, reserpine and insulin; however, tolerance appears to develop upon prolonged exposure (several weeks). Tyrosine hydroxylase, dopamine β-hydroxylase and catecholamine levels are all elevated during chronic exposure, and storage vesicles with a defective uptake system are detectable.

2. Previous work by others:

A vast literature exists on the effects of drugs on central and peripheral catecholamine disposition, and this discussion will be limited to those concerning the development of the adrenal medulla and the actions of drugs on developing adrenergic tissue.

During prenatal and postnatal development, there is a marked increase in catecholamine levels in adrenergic neurons, and in the adrenal medulla, as well as changes in the levels of catecholamine synthesizing enzymes (Ignarro and Shideman, 1968; Heggeness et al., 1970; Iversen et al., 1967; Hökfelt, 1951; Mirkin, 1972; Patrick and Kirshner, 1972). In chick embryos, tyrosine hydroxylase, the rate-limiting enzyme in catecholamine formation, is present on the first day of gestation, and all the enzymes are present by the sixth day. Despite the presence of the necessary enzymes, adrenal catecholamines do not begin developing until several days before birth in the rat (gestational period = 22 days); electron microscopy of adrenal tissue reveals the presence of a small number of storage vesicles four days before birth, with the subsequent appearance (two days before birth) of catecholamines (Daikoku et al., 1969; Elfvin, 1967). These data

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suggest that the presence of storage vesicles is a determining factor in the increase in adrenal catecholamines.

Because the storage vesicles appear relatively late in gestation, the largest changes in catecholamine content occur in the postnatal period. At this stage, neural input plays a role in determining the rate of development in amine stores. Thus, prior sectioning of the splanchnic nerve decreases the rate of development of adrenal catecholamines as well as the age-dependent increases in tyrosine hydroxylase (Patrick and Kirshner, 1972); in sympathetic ganglia, development of catecholamine biosynthetic enzymes is retarded by chronic administration of ganglionic blocking agents (Black, 1973). Neural input similarly accelerates the rate of recovery of amines in adult rats whose stores have been depleted by reserpine treatment (Patrick and Kirshner, 1971a,b). The post-natal dependence on neural input for development of amine stores is paralleled by the development of neurally-dependent response to stress. For example, secretion of catecholamines in response to asphyxia is largely independent of neurogenic stimulation in fetal calves, but is highly nerve-dependent two to three weeks after birth (Comline and Silver, 1966). This indicates that the development of catecholamines is accompanied by fundamental changes in the ability of the animal to utilize the amine stores.

Differences between adult and neonatal catecholamine stores are also apparent in their susceptibility to modification by drug treatment. Infant rats are more sensitive to the catecholamine depleting effects of reserpine and tetrabenazine (Kulkarni and Shideman, 1966). In addition, in chicks hatched from reserpine-treated eggs, following the initial depletion there is a long-lasting (weeks) increase in brain catecholamines, suggesting that alteration of catecholamine levels during development produces a prolonged alteration of catecholamine synthesis and/or storage systems (Sparber and Shideman, 1970). Additionally, drugs which do not ordinarily cross the blood-brain barrier in adults are often able to do so in fetal or neonatal animals (Liuzzi et al., 1974) with the result that peripherally active agents (such as guanethidine or 6-hydroxydopamine) administered systematically produce marked effects in the developing brain which are totally different from the lack of activity in mature brain. Surprisingly, almost no work has been done on the actions of nicotine on adrenergic development on the biochemical level.

The developmental aspects of catecholamines in central and peripheral adrenergic neurons appear to be quite similar to those of the adrenal medulla (Coyle and Axelrod, 1971, 1972a,b; Sachs, 1970), with the largest increases in catecholamines, enzymes and synaptosomal uptake mechanisms occurring in the first few weeks after birth in rodents and chicks. The maturational increases in catecholamines appear to follow the appearance of soluble vesicular proteins (Mirkin, 1974), indicating that the vesicles may also play a determining role in neuronal development. However, most studies of drug effects on adrenergic development have concentrated only on measuring amine levels, and in some cases, tyrosine hydroxylase and dopa decarboxylase activities. Thus, while a number of drugs (nicotine, chlorpromazine, iproniazid, reserpine, morphine, amphetamine, catecholamines, etc.) have been shown to produce long-lasting or permanent alterations in norepinephrine levels in various brain parts, in most of these cases experimental evidence for a specific biochemical lesion has not been

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obtained, nor have any experiments been conducted to determine whether there are functional alterations in catecholamine disposition (synthesis, uptake, storage, release) (Vernadakis, 1974; Liuzzi et al., 1974). For instance, prenatal administration of chlorpromazine to rats shortly before birth leads ultimately to an increased brain norepinephrine level in adulthood (Nair, 1974), but the increases could represent increased synthesis, a greater storage capacity, enhanced uptake, reduced degradation, or reduced release. Administration of guanethidine to newborn mice results in long-lasting reductions of brain catecholamines, but again the mechanism or functional consequences of these developmental changes have not been determined (Liuzzi et al., 1974). Furthermore, no studies have been conducted to see whether the effects of drugs on adrenergic development can be prevented by pretreatment with blocking agents. Obviously, a detailed biochemical profile of enzymes, storage vesicles, uptake and release is required to elucidate the effects of drugs on adrenergic maturation.

(b) Methods

Drug dosage, adult rats.

Sprague-Dawley rats (Zivic-Miller) will be injected subcutaneously twice daily with aqueous solutions of nicotine (1 or 10 mg/kg) or nicotine in oil (2 or 20 mg/kg once daily). In some cases, to differentiate direct vs. reflex effects, rats in acute studies will be pretreated with chlorisondamine (10 mg/kg, s.c.), which blocks neural stimulation of the adrenal medulla; for chronic studies, reflex effects will be prevented by prior sectioning of the left splanchnic nerve while the contralateral adrenal remains innervated to serve for comparison of direct (denervated) vs. direct + reflex (innervated) effects. Animals will be sacrificed at intervals of several hours to several weeks, as well as in the post-exposure period after discontinuing drug administration.

Studies also will be carried out in vitro to determine whether there is any direct effect on catecholamine biosynthetic or degradative enzymes (tyrosine hydroxylase, dopa decarboxylase, dopamine β-hydroxylase, monoamine oxidase) or whether they affect uptake or storage of catecholamines in isolated adrenal medullary vesicles. These studies will identify the direct and indirect biochemical effects of nicotine in mature animals and will thus serve as a model with which to compare drug activities in developing rats.

Drug dosage, developing rats.

In general, the dosages administered to pregnant rats (to expose the offspring in utero) may have to be somewhat lower than in non-pregnant adults, since the LD_{50} of many agents appears to be reduced. In other experiments, normal or prenatally exposed neonates will be given nicotine acutely or chronically either by direct administration or via the milk during chronic administration of the drug to the mother. In the latter experiments, additionally we will differentiate between purely prenatal and purely postanatal effects of nicotine by switching at birth prenatally-exposed pups to normal mothers and normal pups to mothers who will continue to receive nicotine.

Offspring will be sacrificed at intervals of several days from

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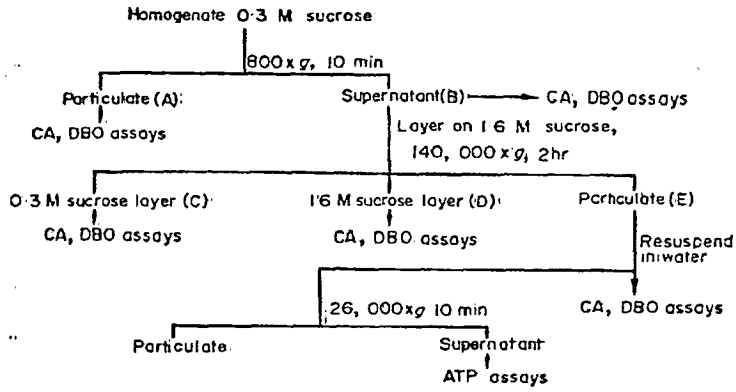
birth to young adulthood (40-50 days). These experiments will determine whether the effects of nicotine are different in developing rats compared to adults and will also indicate whether nicotine affects the biochemical development of catecholamine stores and whether permanent changes in catecholamine disposition can be produced by exposure to nicotine early in development.

Data collection

(a) Determination of the number and contents of storage vesicles:

The adrenal glands from each animal are excised, cleaned of fat and connective tissue, and homogenized (glass-to-glass) in 2.5 ml of ice-cold sucrose-Tris [300 mM sucrose containing 25 mM Tris (pH 7) and 0.1 mM iproniazid (to irreversibly inhibit monoamine oxidase)]. The suspension is centrifuged at 800 g for 10 min and the supernatant is decanted. The pellet is resuspended by glass-to-glass homogenization in 5 ml of distilled water and analyzed for catecholamines and dopamine β-hydroxylase (fraction A). One ml of supernatant is added to 1 ml water, homogenized and assayed (fraction B). Another ml of the 800 g supernatant is layered over 2.5 ml of 1.6 M sucrose containing 500 units/ml of beef catalase (Sigma) and centrifuged for 2 hr at 140,000 g in the No. 40 rotor of the Beckman model L5 ultracentrifuge. This separates intact heavy vesicles from light broken vesicle membranes and from most mitochondrial and lysosomal contaminants (Smith and Winkler, 1967). The 0.3 M sucrose layer (fraction C) and the 1.6 M sucrose layer (fraction D) are diluted with water to final volumes of 2 and 4 ml, respectively, homogenized, and assayed for catecholamines (CA) and dopamine β-hydroxylase (DBH). The vesicular pellet (fraction E) is resuspended in 2 ml water and homogenized to lyse the vesicles. One ml is removed for the determination of CA and DBH, and the remainder is centrifuged at 26,000 g to remove the vesicle membranes; the supernatant of this latter centrifugation is analyzed for ATP. A flow sheet of this procedure appears below.



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DBH is an enzyme associated only with storage vesicles (Viveros et al., 1969a), and the determination of the distribution of DBH activity therefore provides a measure of the number of storage vesicles present as intact heavy vesicles, light vesicles and broken vesicle membranes. The low levels of DBH present early in development require the pooling of glands from several animals to obtain sufficient enzyme activity.

The ratio of catecholamines/DBH provides a measure of the sequence of synthesis of vesicles: new vesicles are deficient in catecholamines and therefore exhibit low ratios. This phenomenon is observed in adult rats in which adrenal stimulation is evoked (Slotkin and Kirshner, 1973a,b) and in developing rats during the stage immediately following the appearance of functional splanchnic innervation (Slotkin, 1973a,b); these data thus provide a sensitive measure for detecting drug-induced alterations in the number and rate of synthesis of vesicles.

ATP is an integral part of the catecholamine storage complex, and drug-induced alterations in ATP indicate changes in catecholamine binding ability. New vesicles and vesicles in developing rats display altered catecholamine to ATP ratios, and this is associated with changes in amine storage (Slotkin and Kirshner, 1973b); the ATP parameter thus provides another sensitive measure with which to compare drug effects in adults and developing rats.

The fraction of vesicles ruptured during homogenization is fairly constant from preparation to preparation (Slotkin and Kirshner, 1973a,b). The distribution of catecholamines and DBH in the broken vesicle fraction thus provide a measure of the mechanical fragility of the vesicles, which is dependent on properties of the vesicle membrane (Slotkin and Kirshner, 1973a,b; Anderson and Slotkin, 1974); the fragility has been observed to change after drug treatments and during development (Slotkin, 1973b; Anderson and Slotkin, 1974).

Alterations in any of the above factors -- catecholamines, DBH, number and distribution of vesicles, ATP levels, vesicle fragility -- can all be used as sensitive, functional indicators of altered catecholamine disposition.

(b) Determination of the uptake and storage capabilities of the vesicles:

One of the major functions of storage vesicles is the ability to incorporate catecholamines by an ATP-Mg^{2+}-stimulated uptake mechanism; non-catecholamines (metaraminol) are incorporated by a non-stimulated mechanism. The uptake mechanisms are known to be altered by nicotine and other drugs, and during the normal maturation process (Lundborg, 1966; Lundborg and Stitzel, 1967; Slotkin and Kirshner, 1973a,b; Slotkin and Edwards, 1973; Slotkin, 1973a; Anderson and Slotkin, 1974), and the evaluation of uptake and storage capabilities are therefore required in the proposed studies.

Adrenal glands from each animal are homogenized in 2.2 ml sucrose-Tris and an aliquot is withdrawn for catecholamine analysis. After centrifugation at 800 g for 10 min, the supernatant is decanted and 0.4 ml is pipetted into each of four tubes containing 0.1 ml of 50 mM ATP plus Mg^{2+}, 0.1 ml of 1 mM epinephrine and either 1 µCi ^{14}C-epinephrine or 5 µCi ^{3}H-metaraminol plus 0.1 ml of 1 mM metaraminol.

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The added epinephrine is sufficient to eliminate any differences in extravesicular amine concentrations among the samples. Sucrose-Tris is added to each tube to give a total volume of 1 ml, and one epinephrine- and one metaraminol-containing sample are brought to 30° for 30 min; the duplicate tubes are kept at 0° for 30 min. Uptake is stopped by the addition of 2 ml of ice-cold sucrose-Tris, and the samples are centrifuged for 10 min at 26,000 g. The supernatants are decanted and added to an equal volume of 7% perchloric acid, centrifuged, and analyzed for CA and radioactivity. The vesicular pellet is washed with sucrose-Tris, centrifuged, rewashed, centrifuged, resuspended (glass-to-glass homogenization) in 3 ml of 3.5% perchloric acid, recentrifuged, and then analyzed for CA and radioactivity. Uptake in each sample is calculated as described below:

$$\text{Gross uptake per gland} = \frac{\text{counts per min in vesicles} \times \text{CA content per gland}}{\text{specific activity of labeling medium} \times \text{CA content of vesicles in sample}}$$

$$\text{Gross uptake per 100 µg CA} = \frac{\text{uptake per gland} \times 100}{\text{micrograms of CA per gland}}$$

The uptake at 0° is then subtracted from the uptake at 30° to give the temperature-dependent vesicular uptakes. Under these conditions, uptake occurs solely into storage vesicles (Slotkin and Kirshner, 1971, 1973b; Slotkin, 1973a).

Because "uptake" is a complex term (influx minus efflux), and since efflux is a measure of the stability of storage, only by the evaluation of efflux can an observed change in uptake be interpreted as altered influx or altered storage. To evaluate efflux, adrenals are homogenized in sucrose-Tris-iproniazid and centrifuged at 800 g. The vesicle-containing supernatant is labeled and washed twice as described above and resuspended in fresh sucrose-Tris. Sets of 15, 1-ml aliquots of labeled vesicles are brought to 37° to allow efflux to occur, and efflux is stopped by the addition of 2 ml of ice-cold sucrose-Tris after 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min. Samples are centrifuged for 10 min at 26,000 g and the supernatant solution is decanted and analyzed for CA and radioactivity. The vesicular pellets are resuspended in 3 ml of 3.5% perchloric acid (to lyse the vesicles), centrifuged 10 min at 26,000 g (to remove precipitated proteins), and analyzed for CA and radioactivity. The effluxes of endogenous CA, ^{14}C-epinephrine and ^{3}H-metaraminol are calculated as described previously (Slotkin et al., 1971).

These methods enable us to evaluate nicotine-induced changes in the development of the uptake and storage of amines in the vesicles.

It should be noted that the uptake of amines into isolated adrenal vesicles is not generally stereospecific (Carlsson et al., 1963). Radioactive dl-epinephrine is used rather than l-norepinephrine because epinephrine is the major catecholamine (80-90%) in the rat adrenal medulla.

(c) Buoyant densities of storage vesicles:

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Catecholamines and nucleotides represent a significant fraction of the dry weight of the storage vesicles (Smith, 1968). Therefore,

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it is not surprising that vesicles with altered contents display altered buoyant densities (Viveros et al., 1969a, 1971a,b; Slotkin and Kirshner, 1973a; Slotkin, 1973b; Anderson and Slotkin, 1974); vesicles with abnormal densities are commonly found in adult rats after administration of catecholamine-depleting agents (Slotkin and Kirshner, 1973b; Slotkin and Edwards, 1973; Anderson and Slotkin, 1974) and in maturation when large numbers of new vesicles appear (Slotkin, 1973b). Consequently, we will examine the effects of nicotine on the buoyant densities of vesicles from adult and maturing rats.

Continuous density gradients, hyperbolic from 1 to 2 M sucrose, are prepared with a modification (Slotkin and Kirshner, 1971) of the apparatus of Ayad et al. (1968). Sucrose (1 M) is pumped into a mixing chamber containing 2 M sucrose (15 ml/gradient) at a rate equal to 30 ml. Adrenal storage vesicles from control rats and from drug-treated rats are labeled with [^{14}C]epinephrine, [^{3}H]metaraminol, or [^{3}H]epinephrine, washed twice, and resuspended in sucrose-Tris. Mixtures of vesicles are prepared as follows: tube 1 contains [^{14}C]-epinephrine (control) + [^{3}H]metaraminol (control); tube 2, [^{14}C]-epinephrine (control) + [^{3}H]metaraminol (drug); and tube 3, [^{14}C]-epinephrine (control) + [^{3}H]epinephrine (drug). The mixtures (total volume, 1 ml/mixture) are then layered onto the gradients and centrifuged at 105,000 x g for 3 hr in a Spinco SW 25 rotor. This is sufficient time to permit equilibrium density to be reached. The gradient tubes are emptied dropwise from the bottom (20 drops/sample), and each sample is diluted with 2 ml of 5% perchloric acid, centrifuged at 26,000 g for 10 min, and analyzed for ^{14}C, ^{3}H, and catecholamines. This method enables detection of small differences in density because both control and drug-treated vesicles are spun in the same gradient.

(d) Development of functional innervation and nicotinic receptors:

Studies with developing rats indicate that functional innervation is lacking in neonates (Slotkin, 1973b). Since one signal for biochemical maturation is the level of neural input to the adrenal (Patrick and Kirshner, 1972; Slotkin, 1973b), it is important to establish whether a given drug accelerates or delays the onset of innervation. This can be tested by administration of 20 IU/kg of insulin s.c. which normally evokes reflex secretion of 30% of total catecholamines in 3 hr in innervated adrenals; if innervation is absent, no secretion occurs (Slotkin, 1973b). This effect is contrasted with the development of nicotinic receptors in the adrenal medulla, as determined by the ability to secrete in direct response to cholinergic drugs. These data will indicate whether nicotine alters the development of splanchnic innervation or of post-synaptic receptors.

(e) Analysis of catecholamine biosynthetic and degradative enzymes:

The development of the ability of the adrenal medulla to synthesize catecholamines generally follows the appearance of tyrosine hydroxylase and dopamine β-hydroxylase (Ignarro and Shideman, 1968; Patrick and Kirshner, 1972; Slotkin, 1973b; Slotkin and Anderson, 1974a). Glands from control or drug-treated adult or developing rats are homogenized in 2.5 ml of 0.15 M KCl and aliquots withdrawn for

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determination of catecholamines, dopamine β-hydroxylase, dopa decarboxylase and monoamine oxidase. The remainder is spun at 26,000 g to sediment catecholamine storage vesicles to avoid interference with the tyrosine hydroxylase assay (26,000 g supernatant). This method enables the determination of catecholamines and all four enzymes from individual rats.

(f) Assays:

For catecholamines, 0.1 ml aliquots of all samples are added to 1.9 ml of 3.5% perchloric acid and centrifuged at 26,000 g x 10 min in order to remove precipitated protein. The supernatants are analyzed for catecholamines by the trihydroxyindole method, using an autoanalyzer (Merrills, 1963).

Radioactive amines are measured by liquid scintillation spectrometry. One milliliter of each sample is added to 10 ml of a 1:2 mixture of Triton X-100 detergent and toluene (containing 2,5-diphenyloxazole and p-bis[2-(5-phenyloxazolyl)]benzene).

Monoamine oxidase activity is measured by the procedure of Laduron and Belpaire (1968), using [^{3}H]tyramine (10 μM) as substrate. Dopamine β-hydroxylase is assayed by the method of Friedman and Kaufman (1965), using [^{3}H]tyramine (10 μM) as substrate. Tyrosine hydroxylase and dopa decarboxylase activity are measured by the method of Waymire et al. (1971), using [^{14}C]tyrosine (100 μM) or [^{14}C] dopa (33 μM) as substrate.

(c) References

Anderson, T.R. and Slotkin, T.A., Biochem. Pharmacol., in press (1974).
Ayad, S.R., Bonsall, R.W. and Hunt, S., Analyt. Biochem. 22:533 (1968).
Black, I.B., J. Neurochem. 20:1265 (1973).
Carlsson, A., Hillarp, N.-A. and Waldeck, B., Acta Physiol. Scand. 59: (Suppl. 215) 5 (1963).
Comline, R.S. and Silver, M., J. Physiol. Lond. 183:305 (1966).
Coyle, J.T. and Axelrod, J., J. Neurochem. 18:2061 (1971).
Coyle, J.T. and Axelrod, J., J. Neurochem. 19:449 (1972a).
Coyle, J.T. and Axelrod, J., J. Neurochem. 19:1117 (1972b).
Daikoku, S., Takashi, O., Takahashi, A. and Sako, M., Tokushima J. exp. Med. 16:153 (1969).
Elfvin, L.G., J. Ultrastruct. Res. 17:45 (1967).
Friedman, S. and Kaufman, S., J. Biol. Chem. 240:4763 (1965).
Heggeness, F.W., Diliberto, J. and DiStefano, V., Proc. Soc. exp. Biol. Med. 133:1413 (1970).
Hökfelt, B., Acta Physiol. Scand. 25:Suppl. 92 (1951).
Ignarro, L.J. and Shideman, F.E., J. Pharmacol. exp. Ther. 159:38 (1968).
Iversen, L.L., deChamplain, J., Glowinski, J. and Axelrod, J., J. Pharmacol. exp. Ther. 157:509 (1967).
Kulkarni, A.S. and Shideman, F.E., J. Pharmacol. exp. Ther. 153:428 (1966).
Laduron, P. and Belpaire, F., Biochem. Pharmacol. 17:1127 (1968).
Liuzzi, A., Foppen, F.H. and Angeletti, P.U., Biochem. Pharmacol. 23: 1041 (1974).
Lundborg, P., Acta Physiol. Scand. 67:423 (1966).
Lundborg, P. and Stitzel, R., Br. J. Pharmacol. Chemother. 29:342 (1967).

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Merrills, R.J., Analyt. Biochem. 6:272 (1963).
Mirkin, B.L., Adv. Behavioral Biol. 8:199 (1974).
Mirkin, B.L., Fed. Proc. 31:65 (1972).
Nair, V., Adv. Behavioral Biol. 8:171 (1974).
Patrick, R.L. and Kirshner, N., Molec. Pharmacol. 7:87 (1971a).
Patrick, R.L. and Kirshner, N., Molec. Pharmacol. 7:389 (1971b).
Patrick, R.L. and Kirshner, N., Devl. Biol. 29:204 (1972).
Sachs, C., Acta Physiol. Scand. Suppl. 341 (1970).
Slotkin, T.A., Biochem. Pharmacol. 22:2023 (1973a).
Slotkin, T.A., Biochem. Pharmacol. 22:2033 (1973b).
Slotkin, T.A., Biochem. Pharmacol., in press (1974a).
Slotkin, T.A., Brit. J. Pharmacol., in press (1974b).
Slotkin, T.A. and Anderson, T.R., Intl. J. Addictive Dis., in press (1974a).
Slotkin, T.A. and Anderson, T.R., Neuropharmacol., in press (1974b).
Slotkin, T.A. and Edwards, K., Biochem. Pharmacol. 22:549 (1973).
Slotkin, T.A. and Green, H.O., Biochem. Pharmacol., in press (1974).
Slotkin, T.A. and Kirshner, N., Mol. Pharmacol. 7:581 (1971).
Slotkin, T.A. and Kirshner, N., Biochem. Pharmacol. 22:205 (1973a).
Slotkin, T.A. and Kirshner, N., Mol. Pharmacol. 9:105 (1973b).
Slotkin, T.A., Ferris, R.M. and Kirshner, N., Mol. Pharmacol. 7:308 (1971).
Smith, A.D., in "The Interaction of Drugs and Subcellular Components in Animal Cells" (P. N. Campbell, ed.), p. 239, Churchill, London (1968).
Smith, A.D. and Winkler, H., Biochem. J. 103:480 (1967).
Sparber, S.B. and Shideman, F.E., Devel. Psychobiol. 3:123 (1970).
Vernadakis, A., Adv. Behavioral Biol. 8:133 (1974).
Viveros, O.H., Arqueros, L., Connett, R.J. and Kirshner, N., Mol. Pharmacol. 5:60 (1969).
Viveros, O.H., Arqueros, L. and Kirshner, N., Mol. Pharmacol. 7:434 (1971a).
Viveros, O.H., Arqueros, L. and Kirshner, N., Mol. Pharmacol. 7:444 (1971b).
Waymire, J.C., Bjur, R. and Weiner, N., Analyt. Biochem. 43:588 (1971).
Waymire, J.C., Vernadakis, A. and Weiner, N., Adv. Behavioral Biol. 8:149 (1974).

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CURRICULUM VITAE

NAME:
Theodore Alan Slotkin

BORN: REDACTED

MARRIED: REDACTED

CHILDREN: REDACTED

SOCIAL SECURITY NUMBER:

EDUCATION AND DEGREES:
B.S. - Brooklyn College, CUNY, 1967
Ph.D. - Department of Pharmacology and Toxicology - University of Rochester, 1970

POSITIONS HELD:

June 1971 - present	Assistant Professor, Department of Physiology and Pharmacology, Duke University Medical Center
June 1970 - May 1971	Research Associate, Department of Biochemistry, Duke University Medical Center
February 1970 - May 1970	Postdoctoral Fellow, Department of Pharmacology and Toxicology, University of Rochester

SOCIETIES:
REDACTED

REDACTED

RESEARCH ACTIVITIES:
Neurochemistry
Neuropharmacology
Hypertension

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BIBLIOGRAPHY

Slotkin, T.A. and DiStefano, V. Urinary metabolites of harmine in the rat and their inhibition of monoamine oxidase. Biochem. Pharmacol. 19:125-131 (1970).

Slotkin, T.A., DiStefano, V. and Au, W.Y.W. Blood levels and urinary excretion of harmine and its metabolites in man and rats. J. Pharmacol. Exp. Ther. 173:26-30 (1970).

Slotkin, T.A. and DiStefano, V. A model of harmine metabolism in the rat. J. Pharmacol. Exp. Ther. 174:456-462 (1970).

Slotkin, T.A. and DiStefano, V. Cardiovascular and respiratory effects of harmine. Proc. Soc. Exp. Biol. Med. 133:662-664 (1970).

Slotkin, T.A., Ferris, R.M. and Kirshner, N. Compartmental analysis of amine storage in bovine adrenal medullary granules. Mol. Pharmacol. 7:308-316 (1971).

Slotkin, T.A. and Kirshner, N. Uptake, storage and distribution of amines in bovine adrenal medullary vesicles. Mol. Pharmacol. 7:581-592 (1971).

Kirshner, N. and Slotkin, T.A. Stimulating experiences with the adrenal medulla. Proc. 8th Midwest Conf. on Endocrinol. and Metabolism. 95-113 (1972).

Slotkin, T.A. and Kirshner, N. All-or-none secretion of adrenal medullary storage vesicle contents in the rat. Biochem. Pharmacol. 22:205-219 (1973).

Slotkin, T.A. and Kirshner, N. Recovery of rat adrenal amine stores after insulin administration. Mol. Pharmacol. 9:105-116 (1973).

Slotkin, T.A. and Edwards, K. The effects of reserpine on the content and properties of rat adrenal medullary storage vesicles. Biochem. Pharmacol. 22:549-560 (1973).

Slotkin, T.A. Maturation of the adrenal medulla. I. Uptake and storage of amines in isolated storage vesicles of the rat. Biochem. Pharmacol. 22:2023-2032 (1973).

Slotkin, T.A. Maturation of the adrenal medulla. II. Content and properties of catecholamine storage vesicles of the rat. Biochem. Pharmacol. 22:2033-2044 (1973).

Slotkin, T.A. and Kirshner, N. Binding of amines to purified bovine adrenal medullary storage vesicle membranes. Biochem. Pharmacol. 22:2492-2497 (1973).

1003546513

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Slotkin, T.A. Hypothetical model of catecholamine uptake into adrenal medullary storage vesicles. Life Sci. 13:675-683 (1973).

Green, H.O. and Slotkin, T.A. Reserpine-like effects of harmine on isolated adrenal medullary storage vesicles. Mol. Pharmacol. 9:748-755 (1973).

Kirshner, N. and Slotkin, T.A. Secretion and recovery of catecholamines by the adrenal medulla. Biochem. Pharmacol. 23: suppl. 351-356 (1974).

Slotkin, T.A. and Green, H.O. Drug-resistant effect of adenine nucleotides and magnesium on catecholamine efflux from isolated adrenal medullary storage vesicles. Biochem. Pharmacol. 23:2190-2192 (1974).

Slotkin, T.A. Structure-activity relationships for the reserpine-like actions of derivatives of β-carboline in vitro. Life Sci., 15:439-454 (1974).

Slotkin, T.A. Reserpine, in "Neuropoisons: Their Pathophysiological Actions" vol. 2 (Simpson, L.L. and Curtis, D.R., eds.). pp. 1-60, Plenum Press, New York (1974).

Slotkin, T.A. and Anderson, T.R. Sympatho-adrenal development in perinatally-addicted rats. Intl. J. Addictive Dis., in press.

Slotkin, T.A. Maturation of the adrenal medulla. III. Practical and theoretical considerations of age-dependent alterations in kinetics of incorporation of catechol- and non-catecholamines. Biochem. Pharmacol., in press.

Slotkin, T.A. and Green, H.O. Adrenal medullary storage vesicles of the spontaneously hypertensive rat. Biochem. Pharmacol. in press.

Anderson, T.R. and Slotkin, T.A. Effects of morphine on the rat adrenal medulla. Biochem. Pharmacol., in press.

Mills, E. and Slotkin, T.A. Catecholamine content of the carotid body in cats after ventilation with 8-100% oxygen. Experientia, submitted.

Slotkin, T.A. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat. Brit. J. Pharmacol., in press.

Slotkin, T.A. and Anderson, T.R. Chronic morphine administration alters epinephrine uptake in rat adrenal storage vesicles. Neuropharmacol., in press.

Slotkin, T.A., Anderson, T.R., Seidler, F.J., and Lau, C. Inhibition of epinephrine and metaraminol uptake into adrenal medullary vesicles by aralkyl- and alkylamines. Biochem. Pharmacol., in press.

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Anderson, T.R. and Slotkin, T.A. Maturation of the adrenal medulla. IV. Effects of morphine. Biochem. Pharmacol., in press.

ABSTRACTS

Slotkin, T.A. and DiStefano, V. Urinary metabolites of harmine in the rat and their inhibition of monoamine oxidase. Fed. Proc. 28:797 (1969).

Slotkin, T.A., DiStefano, V. and Au, W.Y.W. Metabolism of harmine in rats and humans. Pharmacologist 11:273 (1969).

Slotkin, T.A. and DiStefano, V. A model of harmine metabolism in the rat. Fed. Proc. 29:678 (1970).

Slotkin, T.A. Efflux of ^{14}C-epinephrine from bovine adrenal medullary granules. Fed. Proc. 30:445 (1971).

Slotkin, T.A. and Kirshner, N. Structure-activity relationships for uptake and storage of amines by isolated bovine adrenal medullary vesicles. Pharmacologist 13:228 (1971).

Slotkin, T.A. and Kirshner, N. Depletion of rat adrenal medullary constituents following insulin. Fed. Proc. 31:521 (1972).

Slotkin, T.A. Uptake of epinephrine and metaraminol by isolated rat adrenal medullary vesicles following insulin administration. 5th Int. Cong. on Pharmacol. 217 (1972).

Slotkin, T.A. Uptake and storage of amines in isolated adrenal medullary vesicles of developing rats. Fed. Proc. 32:783Abs (1973).

Slotkin, T.A. Maturation of Adrenal Catecholamine Storage Vesicles of the Rat. Pharmacologist 15:210 (1973).

Kirshner, N. and Slotkin, T.A. Recovery of catecholamine stores following secretion by the adrenal medulla. Life. Sci. 13: lxxxiv-lxxxv (1973).

Slotkin, T.A. and Green, H.O. Adrenal Medullary Vesicles of Hypertensive Rats. Clin. Res. 22:13A (1974).

Anderson, T.R. and Slotkin, T.A. Acute and Chronic Effects of Morphine on Rat Adrenal Medulla. Fed. Proc. 33:511 (1974).

Anderson, T.R. and Slotkin, T.A. Effects of morphine on the adrenal medulla of developing rats. Pharmacologist 16:258 (1974).

Slotkin, T.A., Seidler, F.J. and Abou-Donia, M.D. Effects of tryptamines on epinephrine uptake into adrenal medullary vesicles. Pharmacologist 16:257 (1974).

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13. Most recent and pertinent articles:

1. Anderson, T.R. and Slotkin, T.A. Maturation of the adrenal medulla. IV. Effects of morphine. Biochem. Pharmacol., in press.

2. Slotkin, T.A. Maturation of the adrenal medulla. III. Practical and theoretical considerations of age-dependent alterations in kinetics of incorporation of catechol- and non-catecholamines. Biochem. Pharmacol., in press.

3. Slotkin, T.A. Maturation of the adrenal medulla. II. Content and properties of catecholamine storage vesicles of the rat. Biochem. Pharmacol. 22:2033 (1973).

4. Slotkin, T.A. Maturation of the adrenal medulla. I. Uptake and storage of amines in isolated storage vesicles of the rat. Biochem. Pharmacol. 22:2023 (1973).

5. Slotkin, T.A. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat. Brit. J. Pharmacol., in press.

6. Slotkin, T.A. and Green, H.O. Adrenal medullary storage vesicles of the spontaneously hypertensive rat. Biochem. Pharmacol., in press.

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Progress Report #4
July 1, 1974 - December 31, 1974

CTR Grant #864
Theodore Slotkin
Department of Physiology and Pharmacology
Duke University Medical Center
Durham, North Carolina 27710

MATURATION OF THE ADRENAL MEDULLA: CATECHOLAMINE STORES IN NORMAL AND HYPERTENSIVE RATS

Earlier progress reports from this project detailed the biochemical maturation of the adrenal medulla in normotensive Wistar rats (NWR) as well as evaluating adrenal catecholamine synthesis, storage and release in adult spontaneously hypertensive rats (SHR), and therefore these results will not be discussed here. As described in progress report number 3, the properties of storage vesicles from SHR suggested that their responses to autonomic drugs might be different from those of NWR. Studies with reserpine have now been completed and will be published shortly (see publications list).

Reserpine administration resulted in a larger initial decline in adrenal catecholamines in spontaneously hypertensive rats (SHR) than in normotensive Wistar rats (NWR); the difference was eliminated by pretreatment with chlorisondamine. Reserpine also produced a larger increase in SHR catecholamines and dopamine β-hydroxylase several days later; chlorisondamine pretreatment did not prevent the increases, although it did slightly slow the increases. Vesicles from SHR or NWR incubated with reserpine in vitro demonstrated equivalent inhibition of $ATP\text{-}Mg^{2+}$-stimulated epinephrine uptake. Recovery of uptake was more rapid in SHR than in NWR after reserpine inhibition, and this was associated with a burst of new vesicle synthesis in the SHR; chlorisondamine pretreatment reduced the number of new, immature vesicles in reserpine-treated SHR. Both SHR and NWR secreted equal proportions of their adrenal catecholamine contents after nicotine administration. These data suggest that the sympatho-adrenal system of the SHR exhibits an enhanced reflex response to reserpine but that reserpine is equally effective in SHR and NWR in producing blockade of vesicular catecholamine transport; these alterations can affect markedly the actions of autonomic drugs in the SHR.

Similar studies are now underway with nicotine and chlorisondamine (a nicotinic blocking agent). In NWR, nicotine evokes a sympatho-adrenal discharge, resulting in catecholamine depletion, tyrosine hydroxylase induction, and formation of immature vesicles. Chlorisondamine blocks many of the effects of nicotine, but itself evokes some reflex activity. The effects of nicotine disappears upon chronic administration. We will evaluate in the near future whether the acute and chronic effects of nicotine and chlorisondamine are different in the SHR.

The above studies suggest a resemblance between the SHR and human labile hypertension - i.e., periods of low sympathetic activity coupled with hyper-responsiveness to reflex stresses. Preliminary results obtained with developing SHR indicate that, prior to 3 weeks of age (pre-

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hypertensive phase) basal sympathetic activity is high (resembling human essential hypertension); these studies will be continued with other sources of support.

An offshoot of this project has been the development of drugs with reserpine-like sympatholytic activity. An additional publication (see list) details further progress made in this area. Two amine uptake mechanisms appeared to operate in isolated adrenal medullary storage vesicles; one site had a high affinity for epinephrine (Km=30 μM) and low capacity (Umax=20 nmols/100 μg of endogenous catecholamines), while the other had a low affinity (Km=2 mM) and a higher capacity (Umax=130 nmols). The low affinity site was non-specific and did not display competitive inhibition by agents which affected the high affinity, stimulated transport system. The high affinity system was inhibited in a purely competitive fashion by a variety of indoleamines and phenethylamines, but the two classes of compounds displayed different structure-activity relationships. Substitution on the α-carbon decreased the abilities of indoleamines to inhibit stimulated epinephrine uptake, but enhanced activity of phenethylamines. Ring hydroxylation reduced, and methoxylation eliminated, the inhibitory activity of tryptamine, but the same substituents markedly enhanced the activity of phenethylamines. Studies of compounds with restricted side-chain conformation indicated that a condensed structure favored activity in indoleamines while an extended chain enhanced inhibition by phenethylamines. Linear alkylamines of 5 or 6 carbon length were also able to inhibit active epinephrine uptake. None of the agents inhibited the non-stimulated uptake of component of metaraminol, which uses primarily the low affinity system. These data suggest that while indoleamines and phenethylamines do compete with epinephrine for attachment to the high affinity transport site in the vesicle membrane, the point of interaction is probably solely at the locus which binds the amine nitrogen; the remainder of the two types of molecule probably bind to at least two different sites adjacent to the N-binding area.

Publications from this project:

1. Maturation of the adrenal medulla. I. Uptake and storage of amines in isolated vesicles of the rat. T.A. Slotkin, Biochem. Pharmacol. 22:2023-2032 (1973).

2. Maturation of the adrenal medulla. II. Content and properties of catecholamine storage vesicles of the rat. T.A. Slotkin, Biochem. Pharmacol. 22:2033-2044 (1973).

3. Binding of amines to purified adrenal medullary storage vesicle membranes. T.A. Slotkin and N. Kirshner, Biochem. Pharmacol. 22: 2492-2497 (1973).

4. Hypothetical model of catecholamine uptake into adrenal medullary vesicles. T.A. Slotkin, Life Sci. 13:675-683 (1973).

5. Reserpine-like effects of harmine on isolated adrenal medullary storage vesicles. H.O. Green and T.A. Slotkin, Mol. Pharmacol. 9:748-755 (1973).

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6. Secretion and recovery of catecholamines by the adrenal medulla. N. Kirshner and T.A. Slotkin, Biochem. Pharmacol., in press.

7. Drug-resistant effect of adenine nucleotides and magnesium on catecholamine efflux from isolated adrenal medullary storage vesicles. T.A. Slotkin and H.O. Green, Biochem. Pharmacol., in press.

8. Maturation of the adrenal medulla. III. Practical and theoretical considerations of age-dependent alterations in kinetics of incorporation of catechol- and non-catecholamines. T.A. Slotkin, Biochem. Pharmacol., in press.

9. Adrenal medullary storage vesicles of the spontaneously hypertensive rat. T.A. Slotkin and H.O. Green, Biochem. Pharmacol., in press.

10. Structure-activity relationships for the reserpine-like actions of derivatives of beta-carboline. T.A. Slotkin, Life Sci., in press.

11. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat. T.A. Slotkin, Brit. J. Pharmacol., in press.

12. Inhibition of epinephrine and metaraminol uptake into adrenal medullary vesicles by aralkyl- and alkylamines. T.A. Slotkin, T.R. Anderson, F.J. Seidler and C. Lau, Biochem. Pharmacol., in press.

13. Acute and chronic effects of nicotine on synthesis and storage of catecholamines in the rat adrenal medulla. T.A. Slotkin and F.J. Seidler, in preparation.

14. Sympatho-adrenal stimulation and inhibition by acute and chronic chlorisondamine administration to rats. T.A. Slotkin, S.M. Schanberg, F.J. Seidler, C. Lau, J. Bartolome, J. Cooke, Biochem. Pharmacol., submitted.

Abstracts:

1003546519

1. Uptake and storage of amines in isolated adrenal medullary vesicles of developing rats. T.A. Slotkin, Fed. Proc. 32:783Abs. (1973).

2. Maturation of adrenal catecholamine storage vesicles of the rat. T.A. Slotkin, Pharmacologist 15:210 (1973).

3. Adrenal medullary vesicles of hypertensive rats. T.A. Slotkin and H.O. Green, Clin. Res. 22:13A (1974).

4. Effects of tryptamines on epinephrine uptake into adrenal medullary vesicles. T.A. Slotkin, F.J. Seidler and M.D. Abou-Donia, Pharmacologist 16:257 (1974).

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#1019 - WENDER

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

January 24, 1975

Grant Application No. 1019

PHARMACOLOGY

To: The committee comprising Drs. Gardner, Meier and Sommers

Subject: Simon H. Wender, Ph.D., University of Oklahoma
New Application No. 1019
"Effect of Scopoletin and of Various "Stress" Conditions on the Activity of Certain Rat Lung Enzymes"

History

An informal inquiry was handled as Case No. 310: The Executive Committee voted to encourage formal application.

Wender was a grantee of the predecessor to CTR, The Tobacco Industry Research Committee, as far back as 1955, in studies involving polyphenols of cigarette smoke and tobacco. On the basis of his continuing good and productive work he was supported for a considerable period. This was terminated in 1970 because of the question of main-stream relevance.

Request

Application No. 1019 requests $23,448.50 for the first of a three year period.

Documents Submitted (attached)

1. Application dated January 13, 1975. (11 pages including C.V.'s of Drs. Wender and Smith).

2. Appendix (9 pages, including title page, Figure and references).

D.S.

DS:wg
Encl.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

JAN 2 2 1975

Application for Research Grant
(Use extra pages as needed)

Date: Jan. 13, 1975

1. Principal Investigator (give title and degrees): Simon H. Wender, Ph. D., Biochemistry, George L. Cross Research Professor

Co-Principal Investigator: Eddie C. Smith, Ph. D., Biochemistry, Professor

2. Institution & address: University of Oklahoma
Address: Department of Chemistry, 620 Parrington Oval, University of Oklahoma, Norman, Oklahoma, 73069

3. Department(s) where research willibe done or collaboration provided: Chemistry Department (Biochemistry Section)

4. Short title of study: Effect of Scopoletin and of Various "Stress" Conditions on the Activity of Certain Rat Lung Enzymes

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

Our overall aim in the proposed research is to learn specific details of the actual interrelationships among scopoletin of cigarette smoke, various "stresses", and enzymes protecting against lung and/or other tissue damage, as well as other enzymes essential in normal metabolism.

Our group at the University of Oklahoma has recently discovered that scopoletin, esculetin, and chlorogenic acid (known components of cigarette smoke) inhibit the activity of control rat lung glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione peroxidase. (See attached copy of a manuscript describing these findings as recently submitted for publication to LIFE SCIENCES.) These three enzymes are the ones that have been postulated by Chow and Tappel (1) for rat lung, and by Paglia and Valentine (2) and Beutler (3) for mammalian (including human) erythrocytes to comprise a protective mechanism against lipid peroxidation which might occur under various "stress" conditions such as exposure to the pollutants ozone, nitrogen dioxide and carbon tetrachloride; and chronic intake of excess ethyl alcohol.

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8. Brief statement of working hypothesis:

The previously mentioned work of Chow and Tappel, of Paglia and Valentine and of Beutler indicates that animals respond to an increase of lipid peroxides during certain "stress" condition by increasing the activity of glutathione peroxidase, which results in increased peroxide catabolism. This is the key feature of their proposed mechanisms. See accompanying figure I in endix for a mechanism summation by Chow and Tappel. Instead of peroxidation occurring during "stress" to produce tissue damage, harmless hydroxy fatty acids are formed by reduction of the lipid peroxides; these can then be metabolized via the normal β-oxidation pathway. The increases in the activity of glutathione reductase and glucose-6-phosphate dehydrogenase are also parts of the protective chain by providing the increased requirement for reduced glutathione and NADPH, respectively.

Since we have discovered that scopoletin, chlorogenic acid, and esculetin inhibit the three enzymes that comprise the above protective mechanism, we venture a preliminary working hypothesis that inhalation into the lung of scopoletin (and/or chlorogenic acid and esculetin) over an extended period of time may result in inhibition of this protective mechanism of figure I and possibly other protective mechanisms, so that these mechanism(s) may not be capable of protecting lung tissue adequately against the lipid peroxidation caused by certain "stress conditions". In healthy animals subjected to such a "stress", but not receiving scopoletin (cigarette smoke), the peroxidation possibly may be averted by the non-inhibited, actually increased, activity of the three enzymes.

9. Details of experimental design and procedures (append extra pages as necessary)

I. A. Comparison of the relative activity and isozyme composition of lung and also liver glucose-6-phosphate dehydrogenase, glutathione reductase, and glutathione peroxidase from rat controls vs. rats chronically under various "stress" conditions (not limited to those producing lipid peroxidation) such as listed below:

1. Rats nutritionally deficient in
   (a) Alpha-tocopherol (Vitamin E, a biological antioxidant aiding in protection against lipid peroxidation)
   (b) Methionine and cysteine (the latter, a constituent of glutathione)
   (c) Thiamine (vitamin B_1) and/or riboflavin (vitamin B_2) and/or niacinamide
   (d) Combinations of a, b, and c.

2. Rats supplied ethyl alcohol in relatively large amounts

3. Rats fed relatively high concentrations of fat (saturated vs. unsaturated)

4. Chronic exposure of rats to an atmosphere containing nitrogen dioxide or carbon tetrachloride

5. Rats subjected to chronic overcrowding

6. Rats subjected simultaneously to two or more of the above conditions

B. Comparison of the effect of scopoletin especially, but also chlorogenic acid and esculeti and also cigarette smoke particulate matter (containing these three compounds) on the activities of the above three enzymes in lung and liver homogenates from control vs. "stress" rats.

Isozymic forms of some enzymes may change qualitatively and/or quantitatively under varying "stress" conditions, and often individual isozymes are affected differently by the same effector compound. Hilf and coworkers (4) have reported recently that normal mammary glands in mice contained two isoenzymes of glucose-6-phosphate dehydrogenase, whereas a third, more rapidly migrating isoenzyme was found in significant levels only in the pre-neoplastic and neoplastic mammary tissues.

It is of interest to mention here - though we cannot state its precise relevance to animal metabolism as yet - that Wender and coworkers have shown that the amount of scopoletin, and especially of its glucoside scopolin, increases enormously in tobacco plants under many "stress" conditions to which the plant is subjected (5).

continued on next page

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1st continuation page of proposal page 2

II. A. Studies on the effect of scopoletin, etc. on the activity of other key animal enzymes, particularly oxidoreductases, and especially $NADP^+$ - utilizing dehydrogenases.

1. $NADP^+$ - specific isocitrate dehydrogenase

2. $NADP^+$ - specific malate dehydrogenase

3. Benzo - (α) - pyrene hydroxylase (using NADPH)

4. Various "mixed-function oxidases" (using NADPH)

5. Others, such as glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, lactate dehydrogenase, hydroxysteroid dehydrogenase, etc.

We venture the guess that probably some of these or other enzymes, in addition to the three above in I, may be involved in protective mechanisms against tissue damage. Possibly some of these may be affected to a substantial degree by scopoletin, thus also significantly affecting the ability of the animal tissue to be protected against damage during certain "stress" conditions. Some of these enzymes are also part of important metabolic pathways; for example, glucose-6-phosphate dehydrogenase of I is also a key enzyme at the beginning of the hexose monophosphate pathway, also called the pentose phosphate pathway. Inhibition of such enzymes by scopoletin may seriously modify normal metabolism. (For example, in some tissues, NADPH, as usually obtained in significant amounts from the glucose-6-phosphate dehydrogenase reaction, is required for steroid biosynthesis, including steroid sex hormones.) Ayers and Tierney (6) have reported that glucose-6-phosphate dehydrogenase activity was approximately twice as high in the supernatant of homogenized lungs of rats exposed to high PO_2 for 7-10 days and conclude that their "results are consistent with the hypothesis that the pentose phosphate pathway protects against high PO_2." Our Oklahoma findings would indicate that scopoletin is capable of inhibiting the activity of the first enzyme of this pathway, namely glucose-6-phosphate dehydrogenase, and hence passage through this pathway would be inhibited.

Our actual experimental approach in the study of the enzymes will be very similar to the established techniques, such as the methods of cell breakage and kinetic assays, currently used in our laboratory and many other laboratories. We have developed these necessary techniques and skills over the past several years with our studies of peroxidases and/or dehydrogenases from numerous sources including plant and human tissue cultures, plants, bacteria and mammalian tissues. Most are described in accompanying reprints of our recent publications.

We have previously found inhibition by scopoletin of the activity of enzymes 1 and 2 above when these dehydrogenases were obtained from tobacco tissue, but we have not yet tested the others.

Cross and coworkers (7) have pointed out the need of increasing NADPH formation from the glucose-6-phosphate dehydrogenase reaction in order to maintain the -SH levels in chronic ozone exposures. There are, of course, other enzymes besides glucose-6-phosphate dehydrogenase which are capable of supplying some NADPH.

Lange (8) has already reported an inhibition of purified rabbit muscle glyceraldehyde 3-phosphate dehydrogenase and yeast alcohol dehydrogenase by cigarette smoke.

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Aryl hydrocarbon hydroxylases are involved in oxidation of certain hydrocarbons, some of which may be carcinogenic.

III. Studies involving various combinations of I, II, and III. Results obtained in I and II will probably determine which ones are tried.

IV. Studies on the activity of glucose-6-phosphate dehydrogenase, glutathione reductase, glutathione peroxidase and other enzymes which may be found to be affected by scopoletin in II above in rats subjected to chronic exposure to cigarette smoke. The rats used will be controls and others under selected "stress" conditions.

We believe that step by step experimental progression of the research projected in I, II, and III will likely yield experimental results that will lead to the later designing of precise, pertinent experiments in IV which should give clearcut information on the actual in vivo situation regarding these enzymes and cigarette smoke.

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REFERENCES CITED IN PROPOSAL

1. Chow, C. K. and Tappel, A. L. 1972. "An Enzymatic Protection Mechanism Against Lipid Peroxidation Damage to Lungs of Ozone-Exposed Rats", LIPIDS 1 518.

2. Paglia, D. E. and Valentine, W. N. 1967. "Studies on the Quantitative and Qualitative Characterization of Erythrocyte Glutathione Peroxidase", J. LAB. and CLIN. MED. 70 158.

3. Beutler, Ernest 1972. "Drug-induced Anemia", FEDERATION PROC. 31 141.

4. Hilf, R., Rector, W. and Abraham, S. 1973. "A Glucose-6-Phosphate Dehydrogenase Isoenzyme Characteristic of Preneoplastic and Neoplastic Mouse Mammary Tissue", J. NATL. CANCER INST. 50 1395.

5. Wender, Simon H. 1970. "Effects of Some Environmental Stress Factors on Certain Phenolic Compounds in Tobacco", Chapter 1 in the book "Recent Advances in Phytochemistry", Vol. 3, Appleton-Century-Crofts, New York.

6. Ayers, L. and Tierney, D. 1971. "High PO_2 and the Pentose Pathway in Rat Lungs", CLINICAL RES. 19 190.

7. DeLucia, A. J., Hoque, P. M., Mustafa, M. and Cross, C. E. 1972. "Ozone Interaction with Rodent Lung: Effect on Sulfhydryls and Sulfhydryl Enzyme Activities", J. LAB. and CLIN. MED. 80 559.

8. Lange, R. 1961. "Inhibiting Effect of Tobacco Smoke on Some Crystalline Enzymes", SCIENCE 134 52.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Adequate research facilities for the proper prosecution of the investigation are available. The Chemistry Department occupied a new connecting addition in 1971. This new addition greatly enhanced our facilities for biochemical research since it contains a room devoted exclusively to the transfer and maintenance of tissue cultures, a large cold room with space for chromatography, adequate facilities and space for small animal research, a radioisotope room, a room to house our preparative ultracentrifuge and superspeed refrigerated centrifuge plus a general all purpose research room. Much of this research will, however, be carried out in the older part of the Chemistry building in two research laboratories which were completely remodeled in 1965. Among pertinent instruments available in the connecting Chemistry Department buildings are Varian Model 635K double beam recording UV-visible spectrophotometer, Beckman spectrometers, Model DK-1 and Model DU with special micro attachments a Hitachi-Perkin Elmer UV-visible spectrophotometer; Phipps and Bird cigarette smoking apparatus; Aminco-Bowman spectrophotofluorometer, complete with recorder and temperature control; Turner fluorimeter; Perkin-Elmer Model 21 double beam infrared spectrophotometer; Varian Associates Model A-60 analytical NMR spectrometer; Reco, GME, and Calbiochem fraction collectors; R&M Model 810R, and other gas chromatographs; numerous chromatocabs, and other paper, thin-layer and column chromatography facilities; Photovolt densitometer for scanning thin-layer chromatograms; numerous ultraviolet lamps, Blak-Ray Model B-100; Beckman scintillation counter; Beckman Model GP continuous flow paper electrophoresis system; Beckman amino acid analyzer; a Beckman preparative ultracentrifuge; and an Hitachi-Perkin-Elmer mass spectrometer.

A full-time instrument maker, electronics technician, and glassblower are available.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached sheets

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
See attached sheets and reprints and reproductions of recently submitted manuscripts for publication.

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R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Simon H. Wender	15 → 25%	REDACTED
Eddie C. Smith	15 → 25%	REDACTED
Technical		
Post-doctorate (to be recruited)	100%	REDACTED
Graduate student research assistant (to be recruited)	50%	REDACTED
	Sub-Total for A	REDACTED
B. Consumable supplies (by major categories)		
Chemical, glassware, etc.		2,000
Animals (rats)		2,500
	Sub-Total for B	4,500.00
C. Other expenses (itemize)		
Fringe benefits for technical help (14,000 x 13.5%)		1,890
	Sub-Total for C	1,890
	Running Total of A + B + C	REDACTED
D. Permanent equipment (itemize):		
None		-0-
	Sub-Total for D	-0-
E. Indirect costs (15% of A+B+C)	E	3,058.50
	Total request	23,448.50

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	4,950.00	2,080.00	-0-	$3,364.35	25,793.35
Year 3	R	5,445.00	2,288.00	-0-	$3,700.95	28,373.95

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Purification and Characterization of Isoperoxidases from Tobacco	submitted to National Science Foundation	asked for $61,550 for the 2 years	July 1, 1975 to June 30, 1977

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Simon H. Wender

Signature Simon H. Wender Date Jan. 13, 197[illegible]

Telephone R - R
Area Code Number Extension

Checks payable to

University of Oklahoma

Mailing address for checks
University of Oklahoma
Office of Research Administration
1000 Asp Avenue, Room 314
Norman, Oklahoma 73069

Responsible officer of institution

Typed Name Raymond D. Daniels

Title Deputy Director, ORA

Signature Raymond D. Daniels Date 17 Jan 75

Telephone R R
Area Code Number Extension

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BIOGRAPHICAL SKETCHES

Principal Investigator: Dr. Simon H. Wender, George L. Cross Research Professor (Biochemistry) Chemistry Department, Norman, and Consultant Professor of Biochemistry, University of Oklahoma School of Medicine, Oklahoma City.

Date of Birth: R

Place of Birth: Dalton, Ga. (U.S.A.)

Present Citizenship: U.S.A.

Educational Experience:

Emory University, Ga., A.B. and M.S. in Chemistry, 1934 and 1935; U. of Minnesota, Ph.D., Biochemistry, 1938.

University of Oklahoma, George L. Cross Research Professor of Biochemistry (a distinguished Professorship) since 1953; prior to that full Professor, 1948-1953; and Associate Professor, 1946-48.

Consultant Professor of Biochemistry, University of Oklahoma School of Medicine, 1968 —.

University of Kentucky, Instructor, then Assistant Professor, 1942-1946. Texas Agricultural Experiment Station, Associate Chemist, 1939-1942.

Argonne National Laboratory, Div. of Biol. and Med. Research, summers 1953 and 1958. (Visiting research associate 1954-1964).

Fields of Present Major Scientific Interest:

Biochemistry and Chemistry of Phenolic Compounds in Plants; Chromatography; and Plant Oxidoreductases.

Other Information:

Dr. Wender has been a member of the Board of Directors of Oak Ridge Associated Universities, Inc., (1961-1967) and past chairman of the Oak Ridge Council. He is the present Councilor from the University of Oklahoma to ORAU.

Dr. Wender is a member of Phi Beta Kappa, Sigma Xi (former President, Okla. Chapter), Phi Lambda Upsilon, Phi Sigma, Alpha Chi Sigma, American Chemical Society (fomer Chairman, Okla. Section and also Lexington, Ky. Section), American Society of Biological Chemists, American Association for the Advancement of Science (Fellow), American Society of Plant Physiologists, Society for Experimental Biology and Medicine, Oklahoma Academy of Science (Fellow), Phytochemical Society of North America (first International President, 1961-1962), Scandinavian Society for Plant Physiology, and American Institute of Biological Sciences.

During the fall 1966-1967 semester, Dr. Wender was on sabbatical leave doing full-time research on plant phenols and tobacco tissue cultured in vitro, with Dr. Folke Skoog, Botany Department, University of Wisconsin.

During the spring 1974 semester, Dr. Wender was on sabbatical leave doing research on plant phenols with Dr. Eric Conn, Department of Biochemistry and Biophysics, University of California at Davis.

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Co-Principal Investigator: Dr. Eddie Carol Smith

Born:

Height: REDACTED REDACTED

Married to:

REDACTED

Children:

Education:

B.S. in Chemistry: University of Kentucky May 1959
Ph.D. in Biochemistry: Iowa State University July 1963

Professional Experience:

Research Associate: Iowa State University under Dr. David Metzler July 1963-December 1963
Post-Doctoral Scholar: UCLA under Dr. Daniel Atkinson January 1964-September 1965
Assistant Professor: Oklahoma University September 1965-September 1969
Associate Professor: Oklahoma University September 1969-September 1974
Full Professor: Oklahoma University September 1974

Professional Societies:

REDACTED

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PUBLICATIONS OF CO-PRINCIPAL INVESTIGATORS

Dr. Wender is author or co-author of more than 170 research publications. These are concerned primarily with the biochemistry and chemistry of phenolic compounds in various fruits, vegetables, and especially in tobacco and cigarette smoke. In the past four years, he has collaborated with Dr. Eddie C. Smith, an experienced enzymologist, on various enzymes whose activity might be affected by phenolic compounds.

The most recent plus selected, pertinent previous publications by each co-principal investigator, and jointly, are listed below:

"The Effect of Certain Naturally Occurring Compounds on the Activity of Rat Lung Glucose-6-Phosphate Dehydrogenase, Glutathione Reductase and Glutathione Peroxidase", by Pickering, Jerry W., Wender, Simon H. and Smith, Eddie C., LIFE SCIENCES, submitted 1975.

"Substrate Specificities and Molecular Weights of Four Isoperoxidases from Tobacco Tissue Culture", by Powell, B. L., Pickering, J. W., Wender, S. H. and Smith, E. C., PHYTOCHEMISTRY, submitted Nov. 1974.

"Effect of Complete Darkness and of Intense Light on Isoperoxidases in Nicotiana Tabacum Tissue", by Leu, S-L. K., Wender, S. H. and Smith, E. C. PHYTOCHEMISTRY, submitted Nov. 1974.

"Scopoletin: Kinetic Nature of Isoperoxidase A_3 Catalyzed Oxidation and Its Possible Relation to the Physiological Action of this Naturally-occurring Growth Effector", by Reigh, D. L., Wender, S. H. and Smith, E. C., PHYSIOLOGIA PLANTARUM, IN PRESS.

"Phenolic Inhibition of Isoperoxidase A_3", by Reigh, D. L., Wender, S. H. and Smith, E. C., TOBACCO SCIENCE 18 88 (1974).

"Scopoletin: A Substrate for an Isoperoxidase from Nicotiana Tabacum Tissue Culture W-38", by Reigh, D. L., Wender, S. H. and Smith, E. C., PHYTOCHEMISTRY 12 1265 (1973).

"Quantitative Studies of Scopoletin in Cigarette Smoke and Tobacco", by Yang, C-H., Nakagawa, Y. and Wender, S., ANALYTICAL CHEMISTRY 30 2041 (1958).

"Effects of Some Environmental Stress Factors on Certain Phenolic Compounds in Tobacco", by Wender, Simon H., Chapter 1 in the book "Recent Advances in Phytochemistry", Vol. 3, Appleton-Century-Crofts, New York, 1970.

"Evidence for Different Glyceraldehyde-3-Phosphate Dehydrogenases in WI-38 and SV 40-Transformed WI-38", by Dunaway, G. A., Jr., and Smith, E. C., INT. J. BIOCHEM. 4 245 (1973).

"A Comparative Study of Some of the Enzymes Involved in Glucose Metabolism of Human Diploid and SV-40 Transformed Diploid Human Cells", by Dunaway, G. A., Jr., and Smith, E. C., CANCER RESEARCH 31 1418 (1971).

"Glucose-6-Phosphate Dehydrogenase from Tobacco Tissue Culture WR-132. I. Inhibition by Scopoletin and Scopolin", by Kajinami, S., Wender, S. H. and Smith, E. C., PHYTOCHEMISTRY 10 1501 (1971).

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APPENDIX

Effect of Scopoletin and of Various "Stress Conditions on the Activity of Certain Rat Lung Enzymes

by S. H. Wender and E. C. Smith

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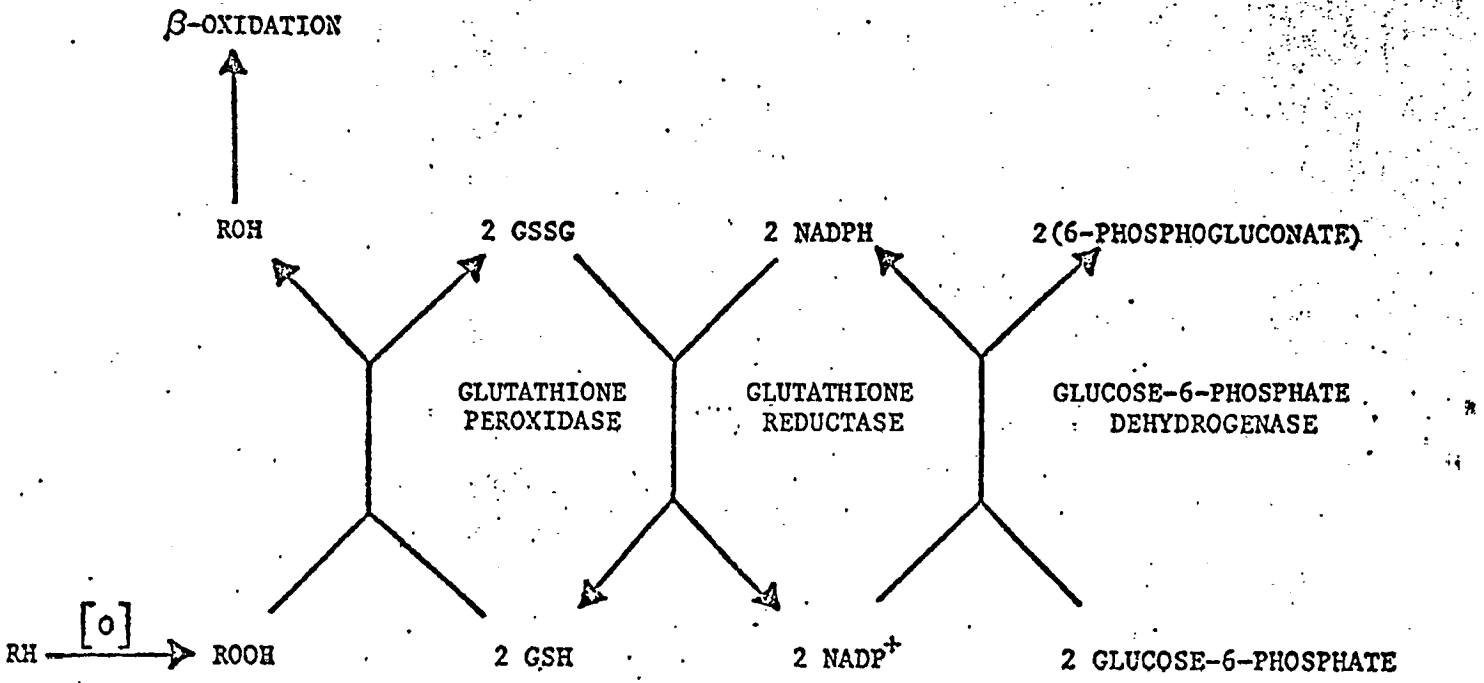
Figure 1



SCHEME OF LIPID PEROXIDE METABOLISM. RH, polyunsaturated fatty acid; ROOH, fatty acid hydroperoxide; ROH, hydroxy fatty acid; GSH, reduced glutathione; GSSG, oxidized glutathione.

Chow, C. K. and Tappel, A. L., Lipids, 1, 518-524. (1972).

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[redacted] manuscript submitted this 1978 to Life Sciences

THE EFFECT OF CERTAIN NATURALLY OCCURRING COMPOUNDS ON THE ACTIVITY OF RAT LUNG GLUCOSE-6-PHOSPHATE DEHYDROGENASE, GLUTATHIONE REDUCTASE AND GLUTATHIONE PEROXIDASE

Jerry W. Pickering, Simon H. Wender and Eddie C. Smith

Biochemistry Section, Chemistry Department, University of Oklahoma, Norman, Oklahoma 73069, U.S.A.

Received:_____

Summary

Nicotine and a number of phenolic compounds known to be components of either cigarette smoke, or tobacco, or both, were tested as possible effectors of the activities of glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase in rat lung homogenates. At a concentration of 0.4 mM, five of these compounds inhibited (up to 90% in some cases) all three of the above enzymes. Other workers have previously proposed that these same three enzymes comprise a protective mechanism in lung against damage by lipid peroxidation.

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Introduction

Glutathione has been shown to reduce potentially damaging lipid peroxides in rat liver homogenates (1). Glutathione peroxidase catalyzed the involved reaction (2). DeLucia et al. (3) have reported that oxidative damage to sulfhydryl (SH) groups in rat lung by prolonged exposure to ozone is counteracted by an increase in the activity of glucose-6-phosphate (G6P) dehydrogenase and a

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corresponding increase in the levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Chow and Tappel (4) have shown that the activities of glutathione peroxidase, glutathione reductase and G6P dehydrogenase are increased significantly in lung homogenates of rats exposed to ozone. They proposed that these enzymes may comprise a protective mechanism to detoxify lipid peroxides resulting from exposure to ozone and other air oxidants. In the present paper, we report the effect of certain individual compounds present in cigarette smoke, or tobacco, or both, upon the activity of the three enzymes included in the above protective mechanism against lipid peroxidation.

Methods and Materials

Enzyme preparation: Lung homogenates were prepared according to a modification of the procedure of Chow and Tappel (4). Mature rats which had been fed a normal diet were sacrificed. Lungs were removed immediately and washed in isotonic KCl. A 20% suspension of the rat lungs in 0.25 M sucrose containing 1 mM EDTA was homogenized at 6,000 r.p.m. for 10 min. in a Sorvall Omnimixer. The homogenate was centrifuged first at 100 x g for 10 min. and then at 105,000 x g for 90 min. to yield a clear red supernatant used for enzyme assays.

Enzyme assays: The assay procedure for glutathione reductase was based upon that of Racker (5). The assay solution contained 0.05 M phosphate buffer (pH 7.6), 0.1 M NADPH, 0.1% BSA and 0.2% oxidized glutathione in a total volume of 3.0 ml. The enzyme reaction was initiated by the addition of 0.1

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ml of the enzyme preparation.

Glucose-6-phosphate dehydrogenase activity was determined by the method of Brown and Wray (6), with the exception that Tris buffer (pH 7.5) was utilized according to the procedure reported for erythrocyte G6P dehydrogenase (7). The assay solution contained 0.02 M Tris-HCl buffer (pH 7.5), 0.3 mM $NADP^+$, 5 mM D-glucose-6-phosphate and 5 mM $MgCl_2$ in a total volume of 3.0 ml. The reaction was initiated by addition of 0.2 or 0.3 ml of the enzyme preparation.

The assay procedure for glutathione peroxidase was that of Paglia and Valentine (8). Commercial glutathione reductase (Sigma) was used in this assay. All effector compounds were prepared in 2% ethanol. An equivalent volume of 2% ethanol was added in control assays.

Results

The effect upon the activities of rat lung glutathione peroxidase, glutathione reductase and G6P dehydrogenase of nicotine and certain phenolic compounds known to be components of either cigarette smoke, or tobacco, or both, is shown in Table I. At a concentration of 0.4 mM, five of these phenolic compounds, namely scopoletin, scopolin, esculetin, esculin and chlorogenic acid, markedly inhibited the activity of glutathione reductase and of G6P dehydrogenase and also substantially inhibited the glutathione peroxidase assay system. None of the other compounds tested exhibited any significant effect upon the activity of any of the three enzymes. However, since the coupled enzyme assay previously used by Paglia and Valentine (8) and which requires glutathione reductase, was used in the glutathione peroxidase assay and since the purified commercial

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Table I

Effects of Phenolic Compounds and Nicotine on Activity of Three Enzymes from Rat Lung

0.4 mM Effector	Glutathione Peroxidase	Glutathione Reductase	Glucose-6-phosphate Dehydrogenase
None	100	100	100
Caffeic acid	100	102	99
Chlorogenic acid	24	33	13
p-Coumaric acid	102	102	100
Esculetin	12	45	68
Esculin	16	45	49
Ferulic acid	101	100	100
Nicotine	102	102	100
Salicylic acid	102	96	100
Scopoletin	14	37	43
Scopolin	17	55	27
Vanillic acid	100	102	100

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glutathione reductase used in the assay system displayed the same pattern of inhibition as the rat lung enzyme, it is not possible at present to determine how much of the observed inhibition can be attributed to the inhibition of glutathione peroxidase. It is speculated that a significant amount of the inhibition is due to glutathione peroxidase because of the excess quantities of glutathione reductase used in the assay; however, quantitative measurements await the use of a direct assay for glutathione peroxidase.

Discussion

Of those phenolic compounds which we have found to have an inhibitory effect on the enzyme systems studied, scopoletin, esculetin, and chlorogenic acid have been previously shown to be present in cigarette smoke. Scopoletin is by far the most abundant of the three in the smoke. In 1958, scopoletin was identified in the mainstream smoke of all 29 different brands of cigarettes tested, some of which had filters (9). The amount of scopoletin present in smoke for each 1 g of tobacco originally present in the smoked cigarette ranged from 10.3 to 27.4 µg. Chlorogenic acid (10) and esculetin (11) have also been identified in trace amounts in cigarette smoke. Scopolin and esculin, which are glucosides of scopoletin and esculetin, respectively, have not been found in cigarette smoke, although they have been reported to be present in tobacco plants (12).

The smoke from cigarettes has been shown by several groups of research workers to inhibit some dehydrogenases. Sato and coworkers (13) have reported that inhibition of succinic dehydrogenase by cigarette smoke was eliminated

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by cysteine or glutathione. Lange (14) reported an inhibition of purified rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and yeast alcohol dehydrogenase by cigarette smoke. The observed inhibition of both of these enzymes was partially overcome by prior inhalation of the smoke. Lange suggested that this inhibitory effect of cigarette smoke might be the result of oxidation of SH groups by peroxides which might be present in the smoke. Subsequently (15), peroxides were identified in cigarette smoke.

Using specific effectors and enzyme system assays, we have found that the phenolic lactone scopoletin, a significant component of cigarette smoke, inhibits glutathione reductase, G6P dehydrogenase, and possibly glutathione peroxidase from rat lungs. These enzymes are the very ones that have been postulated by Chow and Tappel (4) to comprise a protective mechanism against lipid peroxidation in mammals.

Acknowledgement

This research was supported in part by a grant from the National Institutes of Health.

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References

1. B. O. CHRISTOPHERSEN, Biochem. J. 100, 95 (1960).

2. B. O. CHRISTOPHERSEN, Biochim. Biophys. Acta 164, 35 (1968).

3. A. J. DeLUCIA, P. M. HOQUE, M. G. MUSTAFA and C. E. CROSS, J. Lab. and Clin. Med. 80, 599 (1972).

4. C. K. CHOW and A. L. TAPPEL, Lipids 7, 518 (1972).

5. E. RACKER, In: Methods in Enzymology, Vol. 2, eds. S. P. Colowick and N. O. Kaplan, p. 722. Academic Press Inc., New York, N.Y. (1955).

6. A. P. BROWN and J. L. WRAY, Biochem. J. 108, 437 (1968).

7. R. G. LANGDON, In: Methods in Enzymology, Vol. 9, eds. S. P. Colowick, and N. O. Kaplan, p. 126. Academic Press Inc., New York (1966).

8. D. E. PAGLIA and W. N. VALENTINE, J. Lab. and Clin. Med. 70, 158 (1967).

9. C-H. YANG, Y. NAKAGAWA and S. H. WENDER, Anal. Chem. 30, 2041 (1958).

10. W. STECK and S. H. WENDER, Tobacco Science, 9, 54 (1965).

11. L. J. DIETERMAN, C-H. YANG, NAKAGAWA, Y. and S. H. WENDER, J. Org. Chem. 24, 1134 (1959).

12. R. L. STEDMAN, Chemical Reviews 68, 153 (1968).

13. T. SATO, T. SUZUKI and T. FUKUYAMA, Brit. J. Cancer 16, 7 (1962).

14. R. LANGE, Science 134, 52 (1961).

15. J. D. TELLER, Abstracts, 150th National Meeting of the American Chemical Society, New York, N. Y. (1965).

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

DEC 3 1974

Application for Research Grant
(Use extra pages as needed)

Date: 11-19-74

1. Principal Investigator (give title and degrees):

Carl G. Becker, M.D., Assoc. Professor of Pathology, Principal Investigator

Gregory Siskind, M.D., Assoc. Professor of Medicine, Consultant in Immunology

2. Institution & address:

Cornell University Medical College
Department of Pathology
1300 York Avenue
New York, New York 10021

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Investigation of the Role of Allergy to Tobacco Constituents in the Pathogenesis of Arteriosclerosis and Myocardial Infarction.

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

Smoking of tobacco and atherosclerosis have been associated statistically (1-4). The pathogenetic mechanisms that are the basis of this relationship are obscure. Research proposed herein is designed to examine the hypothesis that certain people become allergic to constituents of tobacco or tobacco smoke and that injury to blood vessels, arteriosclerosis, and injury to the myocardium may be consequent to repeated challenge with tobacco allergens (5-7). Scarring of pulmonary blood vessels has been observed with greater frequency in lungs of cigarette smokers than in lungs of pipe or cigar smokers or non-smokers (8).

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In the course of these investigations we propose to:

a) Isolate, purify and identify as allergenic, material from tobacco. We propose to identify the route of entry and absorption of this allergenic material.

b) Examine sera from non-smokers and smokers with and without clinical evidence of ischemic heart disease and with and without pulmonary emphysema for the presence of reaginic (IgE) antibodies

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Research Aims (cont'd)

to tobacco antigens by radioimmunoassay technique. We propose to skin test the same subjects for immediate and delayed hypersensitivity to these antigens. Because of recent data indicating that in rabbits production of anaphylactic or IgE antibodies can be especially stimulated by neonatal immunization, we propose to examine the sera of children from smoking and non-smoking mothers for IgE antibodies to tobacco antigens. If possible, we shall also skin test these children.

8. Brief Statement of Working Hypothesis:

The hypothesis underlying the proposed research is that some tobacco smokers become sensitized to one or more allergenic components of tobacco smoke and that repeated challenge with these allergens by smoking can result in immunologic injury to blood vessels, accentuating the development of athero-arteriosclerosis and increasing the risk of myocardial infarction. Similarly, repeated immunologic injury to pulmonary blood vessels may contribute to the development of pulmonary emphysema. A related, additional hypothesis is that some smokers sensitized to tobacco allergens can develop myocardial anaphylaxis on repeated contact with these allergens.

9. Details of Experimental Design:

A. Purification and Identification of Tobacco Allergens:

1) Tobacco Leaf (Glycoprotein (TGP) --

We have extracted cured tobacco leaves of Maryland, Turkish, Burley, and Flue cured varieties (obtained through the courtesy of American Tobacco Co.) with phosphate buffered saline, pH 7.4 (PBS) after delipidization with petroleum ether. The saline extracts were brought to 60% saturation with crystalline $(NH_4)_2SO_4$, redissolved in PBS and applied to G-25 Sephadex columns from which it emerged with the void volume as a single, sharp, symmetrical peak. When this peak was applied to 7.5% polyacrylamide gels, pH 8.9 and subjected to electrophoresis it migrated rapidly and anodally as a single, brown band which stained with both protein stains (Amido black or Coomassie blue) and the PAS reaction, indicating that it is a glycoprotein (Fig. 1).

The electrophoretic migration and staining characteristics of material extracted from the four different kinds of tobacco were identical (Fig. 2). This purified material, when injected intradermally induced wheal and flare reactions within 30 minutes of intradermal injection in three of six smokers and in one non-smoker with a history of rhinorrhea in response to tobacco

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Details of Experimental Design (cont'd)

smoke exposure. It produced no reaction in two of two non-smokers. This material is presumably the allergenic principle present in tobacco extracts described by Harkavy and Perlman (5). For purposes outlined in this research proposal tobacco leaf glycoprotein antigen (TGP) will be prepared as outlined above.

2) Tobacco Leaf Lipids --

a) Rhus toxin is volatilized when leaves of the genus Rhus (poison ivy, poison oak, poison sumac) are burned, yet still capable of inducing contact dermatitis by mechanisms related to delayed hypersensitivity in sensitive subjects. We propose to examine the lipid soluble material from cured tobacco leaves for allergenic material that on burning become volatilizable haptens.

b) The petroleum ether extract of cured tobacco leaves that is obtained from the first stage of preparation of TGP will be concentrated by evaporation of the petroleum ether. The resulting material will be assayed for allergenicity as described below.

B. Demonstration and Characterization of Antibodies to Tobacco Leaf Glycoprotein (TGP) in Human Serum:

1) The purpose of this phase of the proposed research is to determine if smokers develop humoral antibodies to TGP, to determine the frequency of immunization, and to determine which class of antibodies are formed.

2) Characterization and quantitation of the antibodies to TGP.

a) TGP will be coupled to Sepharose beads activated with cyanogen bromide to prepare an insoluble immunoabsorbent (S-TGP) (9).

b) Aliquots of serum from subjects described above will be incubated with S-TGP and then centrifuged and washed several times.

c) After step (b), aliquots of S-TGP will then be incubated with I^{125}-labeled antibodies specific for heavy chains of IgE, IgG, IgA and IgM. After centrifugation and washing, the radioactivity will be counted and the quantity of immunoglobulin specifically bound to S-TGP calculated from previously prepared standard curves.

d) If it is demonstrated that some smokers have significantly elevated levels of IgE antibodies specifically directed against TGP and other smokers or non-smokers have no IgE anti-TGP we propose to test volunteers from both groups for immediate hypersensitivity to TGP by intradermal injection of TGP in doses of 10 and 50 µgrams. This will serve as a bioassay control for the radioimmunoassay of IgE antibodies specific for TGP.

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Details of Experimental Design (cont'd)

3) The radioimmunoassay procedure described above will be used to examine serum samples obtained from the following groups of people for antibodies to TGP:

a) smokers without evidence of ischemic heart disease,

b) age-matched smokers with evidence of ischemic heart disease,

c) age-matched non-smokers,

d) patients with thromboangiitis obliterans,

e) smokers with clinical evidence of emphysema

f) non-smokers with clinical evidence of emphysema or other chronic pulmonary disease,

g) smoking and non-smoking medical students,

h) former smokers who have given up smoking.

We would begin by studying blood samples from our medical student class from whom we will also obtain a history of smoking habits and of allergies. These serum samples will be used to establish the assay technique as well as to obtain data on our student population. Once the radioimmunoassay procedures are found to be satisfactory we will study serum samples from the other patient groups. In this connection I have been promised the help of our Division of Cardiology in obtaining serum samples from patients with ischemic heart disease or peripheral vascular disease. Similarly, the physicians staffing our pulmonary clinic have also expressed great interest in this project and have promised cooperation in obtaining serum samples from patients with chronic pulmonary disease. Finally, Dr. Lawrence Hinkle, Director of the Cornell Human Ecology Program, studying events contributing to myocardial infarction and sudden death in employees of the Telephone Company has agreed to provide us with serum specimens from human subjects in this study. Extensive data on smoking and dietary habits as well as physical examinations, measurements of serum cholesterol, etc, have been collected on these patients. We will have an opportunity to compare smokers, non-smokers, and former smokers with respect to antibodies to tobacco antigens. These patients have also been evaluated for presence or absence of clinical evidence of arteriosclerotic cardiovascular disease. If we can demonstrate that some smokers are indeed immunized to tobacco products, we can then compare a population of smokers with and without clinical evidence of arteriosclerotic cardiovascular disease with respect to the presence or absence of antibodies to tobacco constituents.

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4) Characterization of the route of absorption of TGP.

a) The question arises as to how TGP is absorbed. Is a hapten released from it when tobacco is burned? Or,

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Details of Experimental Design (cont'd)

is TGP taken in with steam generated behind burning tobacco? In this connection, an allergenic muscle protein has been demonstrated in the steam from cooking codfish (10). We propose to examine saliva collected from smoking subjects and condensates of tobacco smoke for the presence of intact TGP or fragments of TGP present as haptens. We also propose to examine the blood of smokers for TGP antigen. A radioimmunoassay using I^{125}-labeled TGP and competitive protein binding technique will be the means of these measurements.

b) Antisera to TGP will be prepared in rabbits. Antibody to TGP will be purified from sera from immunized rabbits using columns of TGP coupled to Sepharose.

c) TGP will be radio-labeled and the capacity of standard anti-TGP sera to bind TGP determined. Antibody will then be incubated with known amounts of cold TGP and then with labeled TGP and a standard curve constructed.

d) Saliva and blood of subjects will be collected before, during, and after smoking. We will look for TGP on saliva and serum samples by measuring the capacity of the samples to inhibit binding of radiolabeled TGP to anti-TGP.

e) If there is evidence of TGP in saliva, a large quantity of saliva will be collected from smoking subjects. In order to see if TGP is present in intact form we shall attempt to reisolate it from saliva and compare it physically and immunochemically with TGP isolated from tobacco leaves.

f) Failure to reisolate intact TGP from saliva containing TGP reactive with anti-TGP would suggest that it is present as a hapten. If so, we shall attempt to isolate the hapten by using anti-TGP coupled to an insoluble matrix.

g) We also propose to examine condensates of tobacco smoke for TGP by radioimmunoassay as well as attempt to isolate intact and/or haptenic TGP from these condensates.

h) It is conceivable that other of the family Solanaceae, i.e. potatoes, tomatoes, green pepper, eggplant, have antigens similar to tobacco and that some people become sensitized consequent to ingesting these antigens. When they smoke, they challenge themselve with similar or cross reactive antigens. To test this hypothesis we propose to extract potatoes, tomatoes, green peppers,

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Details of Experimental Design (cont'd)

and eggplant to see if we can demonstrate antigen similar to TGP. Glycoprotein extracted from these sources will be compared with TGP by double diffusion in agarose gel against rabbit antisera to highly purified TGP. If in the course of experiments described herein, other antigens are obtained from tobacco to which smokers are allergic, we shall see if these antigens are also distributed among the other Solanaceae.

C. Assay of Tobacco Leaf Lipid (TLL) for Allergenic Activity:

1) It is not known whether any of the lipid soluble material in tobacco leaf is allergenic. In fact, in two recent publications dealing with the chemical nature of allergens, there is no mention of allergenic lipids (11,12).

2) The crude lipid extract will be prepared as described above. Since this is a complex mixture of unknown properties we will assay it for allergenicity by indirect means rather than by skin testing human subjects.

3) Some subjects allergic to tobacco glycoprotein (TGP) might well be allergic to TLL as well. Serum from subjects with high levels of IgE antibodies to TGP and serum from subjects with no antibodies to TGP will be injected intradermally into a Rhesus monkey. After 50 hours and immediately following systemic injection of Coomassie blue, the sites of serum injection will be injected with TLL (Prausnitz-Kustner (P-K) reaction) and observed for blueing. Alternatively, the TLL will be injected intravenously and the sites of serum injection observed for blueing (Passive Cutaneous Anaphylaxis (PCA) reaction). The TLL may not be soluble in saline, in which case we would extract TLL with the monkey's own serum in the hope that some of the lipid components might bind to serum albumin and/or lipoproteins. The TLL serum extract would then be used for P-K or PCA reactions.

4) Blood will be drawn from subjects with and without reaginic antibodies to TGP and the capacity of TLL to stimulate histamine release from their leukocytes measured (13).

5) If the lipid extracts are shown to have allergenic activity, we propose to fractionate the lipid extract to isolate the specific allergen(s) using techniques of thin layer chromatography on silica gels, solvent resistant Sephadex, etc.

6) Lipid fractions obtained in paragraph (5) would be assayed as in paragraphs (3) and (4) for allergenic activity.

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Details of Experimental Design (cont'd)

7) Purified lipid fractions demonstrated to have allergenic activity will be coupled to insoluble matrices and human sera will be examined for antibodies to them by radioimmunoassay technique as described above for TGP. If the lipid fractions are nontoxic we would also attempt skin testing of human subjects.

8) If it can be shown that certain lipid components of tobacco leaf are allergenic we would like to examine condensates of tobacco smoke for their presence, employing techniques of thin layer chromatography or gas liquid chromatography. Lipids recovered from tobacco smoke and corresponding chromatographically to those isolated from tobacco leaf will be assayed for allergenicity as described above.

References

1. Auerbach O, Hammond ED and Garfinkel L: Smoking relation to atherosclerosis of the coronary arteries. NEJM 273:2686, 1965.

2. Strong JP, et al: On the association of cigarette smoking with coronary and aortic atherosclerosis. J Athero Res 10: 303, 1969.

3. Sackett DL, et al: Relation between aortic atherosclerosis and the use of cigarette and alcohol. NEJM 279:1413, 1968.

4. Hatch FT, et al: A study of coronary artery disease in young men: Characteristics and metabolic studies of the patients and comparison with age-matched young men. Circ 33:679, 1966.

5. Harkavy J and Perlman E: Tobacco allergy in coronary artery disease. NY State J of Med 64:1287, 1964.

6. Harkavy J: Cardiac manifestations due to allergy. NY State J of Med 64:2289, 1964.

7. Harkavy J: Cardiovascular manifestations due to hypersensitivity. NY State of Med 69:2757, 1969.

8. Auerbach O, et al: Smoking habits and age in relation to pulmonary changes: rupture of alveolar septums, fibrosis, and thickening of walls of small arteries and arterioles. NEJM 269:1045, 1963.

9. Axen R, Porath J, and Sverker E: Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature 214:1302, 1967.

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Details of Experimental Design (cont'd)

10. Aas K and Jebsen JW: Studies of hypersensitivity to fish: Partial purification and crystallization of a major allergenic component of cod. Int Arch Allergy 32:1, 1967.

11. Berrens L: The chemistry of atopic allergens. Monographs in Allergy, vol 7. S Karger, New York, 1971.

12. Stanworth DR: Immediate hypersensitivity. The molecular basis of the allergic response. American Elsevier Publishing Co, New York, 1973.

13. May CD: Procedures for immunochemical study of histamine release from leukocytes with small volumes of blood. J Allergy 46:12, 1970.

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10. Space and Facilities Available:

1) Laboratory Space --

Four large laboratories with hoods for analytical chemistry, lipid chemistry, histochemistry, and processing tissues for electron microscopy.
One small laboratory for bacteriology.
One small laboratory for immunochemistry and protein chemistry.
One small laboratory for ultramicrotomy for electron microscopy.
One small utility room for inoculations, bleeding of rabbits, preparation of special diets, and autopsy of animals.
One utility room with autoclave, automatic glassware washer, drying oven, and distillation apparatus.
Two 4°C cold rooms for protein chemistry and storage of special diets.
One room for tissue culture. This room is equipped with a small cubicle for sterile transfer, a CO_2 incubator, and an inverted microscope.

2) Offices for each investigator and a technician/secretary.

3) Animal Quarters --

One large animal room with 40 breeding cages and 150 other cages for maintenance of specially bred stock of rabbits and other experiments.
One small room for isolation and maintenance of approximately 50 rabbits receiving streptococcal infections.
In addition, this room has 15 metabolism cages for collection of urine from infected rabbits.

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Space and Facilities Available (cont'd)

One small animal room with cages for 80 rabbits fed lipid-rich diets.
One small animal room with cages for approximately 40-50 rabbits with foreign serum induced hypertension. In addition, this room contains 9 metabolism cages for collection of urine from hypertensive and nonhypertensive rabbits. (It also contains cages for approximately 10 guinea pigs for immunization).
One small room for storage of food, shavings, and cleaning equipment for the animal rooms.
One small room with cage cleaning facilities.
Three refrigerators - 2 for storage of dead animals and 1 for storage of special diets.

4) One Siemens Elmiskop I and one RCA EMU 3-B electron microscope with two dark rooms, one for developing plates and one for printing (departmental facility shared with other investigators).

5) A scanning electron microscope, ETEC Ultrascan, has recently been acquired as an institutional instrument and is readily available for the use of Dr. Minick and Dr. Insull one day per week. In addition, there is considerable evening and weekend time available. The facility includes a dark room, a critical point dryer, vacuum evaporator and stereoviewer. A cryostage and micromanipulator are to be delivered.

6) Facilities for ultramicrotomy, including one Porter Blum MT-1 ultramicrotome, one LKB Ultratome with special cutting table, one LKB knife maker, one dissecting microscope, and an oven for polymerization of embedding materials.

7) Histology laboratory facilities, including 2 American Optical microtomes, one knife sharpener, staining equipment, and paraffin oven. A Technicon is shared with the departmental histology laboratory.

8) One Beckman DB spectrophotometer equipped with automatic wavelength programmer and potentiometric recorder and 1 Coleman junior spectrophotometer.

9) One refrigerated high speed centrifuge, Beckman LB75 preparative ultracentrifuge, and 3 table centrifuges.

10) Electrophoresis equipment. Buchler apparatus for agar gel immunoelectrophoresis, analytical polyacrylamide electrophoresis and preparative polyacrylamide electrophoresis. Equipment for starch gel, starch block and agarose block electrophoresis.

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Space and Facilities Available (cont'd)

11) Equipment for column chromatography including 2 fraction collectors (one fraction collector is very worn and must be replaced).

12) One bacteriology incubator, 3 water baths, and 1 constant temperature bath. Two ovens for drying and evaporation.

13) One Braun disintegrator for grinding bacteria.

14) Two distilling apparatuses, one for distillation of lipid solvents and one for double distilling water.

15) Three deep freezers and four refrigerators.

16) One vacuum-freeze lyophilizer.

17) One IEC cryostat. (The latter is departmental equipment shared with others).

18) One analytical balance, three torsion balances, one beam balance and two scales for weighing rabbits and feed. The torsion balance in the animal room is over 10 years old and badly worn and must be replaced.

19) One Grass model 5P1 polygraph with four channels. One strain gauge pressure transducer. (This equipment is on loan from another department, must be returned, and a replacement purchased.)

20) Two pH meters.

21) Four microscopes and 2 photomicroscopes (both adapted for fluorescence microscopy).

22) Photographic apparatus for photographing gross pathology specimens.

23) One Nuclear Chicago Gamma Radiation Counter, 1185 series.

24) One Nuclear Chicago Liquid Scintillation Counter, Isocap 300.

11. Additional Facilities Required:

None

12. Biographical Sketches of Investigators:

Curricula of Dr. Becker and Dr. Siskind are appended.

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13. Pertinent Publications:

Reprints of the 15 publications listed below that are pertinent to this application have been supplied earlier (July 15th, 1974) in triplicate to the offices of The Council for Tobacco Research.

1) Minick CR, Murphy GE and Campbell WG Jr: Experimental Induction of Athero-arteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. I. Effect of repeated injections of horse serum in rabbits fed a dietary cholesterol supplement. J Exp Med, 124, no 4, 635-652, 1966.

2) Minick CR and Murphy GE: Experimental Induction of Atheroarteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. II. Effect of repeatedly injected foreign protein in rabbits fed a lipid-rich, cholesterol-poor diet. Am J Path, 73, no 2, 265-300, 1973.

3) Hardin NJ, Minick CR, and Murphy GE: Experimental Induction of Atheroarteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. III. The role of earlier acquired fibromuscular intimal thickening in the pathogenesis of later developing atherosclerosis. Am J Path, 73, no 2, 301-326, 1973.

4) Becker CG and Murphy GE: Demonstration of Contractile Protein in Endothelium and Cells of the Heart Valves, Endocardium, Intima, Arteriosclerotic Plaques, and Aschoff Bodies of Rheumatic Heart Disease. Am J Path, 55, no 1, 1-22, 1973.

5) Becker CG and Nachman RL: Contractile Proteins of Endothelial Cells, Platelets and Smooth Muscle. Am J Path, 71, no 1, 1-22, 1973.

6) Becker CG: Demonstration of Actomyosin in Mesangial Cells of the Renal Glomerulus. Am J Path, 66, no 1, 97-110, 1972.

7) Jaffe EA, Nachman RL, Becker CG and Minick CR: Culture of Human Endothelial Cells Derived from Umbilical Veins. Identification of morphologic and immunologic criteria. J Clin Invest, 52, no 11, 2745-2756, 1973.

8) Levi R: Hypersensitivity Reactions of the Heart: An Experimental Model. Bul NY Acad Med, 46, no 11, 997, 1970.

9) Levi R: Effects of Exogenous and Immunologically Released Histamine on the Isolated Heart: A Quantitative Comparison. J Pharm and Exp Therapeut, 182, no 2, 227-238, 1972.

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Pertinent Publications (cont'd)

10. Capurro N and Levi R: Anaphylaxis in the Guinea-Pig Isolated Heart: Selective inhibition by burimamide of the positive inotropic and chronotropic effects of released histamine. Brit J Pharm, 48, no 4, 620-628, 1973.

11. Levi R and Capurro N: Histamine H_2-Receptor Antagonism and Cardiac Anaphylaxis. Proc Intl Symp on Histamine H_2-Receptor Antagonists, Eds CJ Wood and MA Simkins, Smith Kline and French, London, 175-183, 1973.

12. Capurro N and Levi R: The Heart as a Target Organ in Systemic Allergic Reactions: Comparison of Cardiac Anaphylaxis *in vivo* and *in vitro*. Submitted for publication to Circulation Research, July 1974.

13. Harkavy J and Perlman E: Tobacco Allergy in Coronary Artery Disease. NY State J of Med, 64, no 11, 1287-1296, 1964.

14. Harkavy J: Cardiac Manifestations Due to Allergy. NY State J of Med, 64, no 18, 2291-2302, 1964.

15. Harkavy J: Cardiovascular Manifestations Due to Sensitivity. NY State J of Med, 69, no 21, 2757-2765, 1969.

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount	Fringe
Professional (give % time of investigator(s) even if no salary requested)			
None			
Technical			
1. Immunochemistry technician	80%	$10,000	$1,575
2. General laboratory and animal technician	70	6,666	1,166
Sub-Total for A		$19,407	

B. Consumable supplies (by major categories)

1. Rabbits in which to make anti-TGP antibody. Two goats for preparation of antibody to human IgE. Approximately 6 Rhesus or African green monkeys for passive sensitization experiments with human sera (Prausnitz-Kustner reactions)	1,400
2. Chemicals, biologicals, isotopes, glassware, disposable test tubes, pipettes, etc. for radioisotope work, stationery	4,000
Sub-Total for B	$5,400

C. Other Expenses (itemize)

Maintenance and service contract costs on centrifuges, counting equipment, etc.	
Sub-Total for C	$1,200
Running Total of A + B + C	$26,007

D. Permanent equipment (itemize)

None

Sub-Total for D	
E. Indirect costs (15% of A+B+C) E	3,901
Total request	$29,908

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Contractile Proteins of Vascular Smooth Muscle*	Am. Heart Assoc. #72-900	$20,365	7/1/74 to 6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount/yr.	Inclusive Dates
Arteriosclerosis, glomerulonephritis and rheumatic fever*	NIH HL-01803	$150,000	7/1/75 to 6/30/80 if approved and funded
Pathogenesis of Post-streptococcal Glomerulonephritis	Am. Heart Assoc.	26,290	7/1/75 to 6/30/78
Contractile and Regulatory Proteins of Blood Vessels and Platelets	N.Y. Heart Assoc.	20,011	7/1/75 to 6/30/77

*Research on tobacco is not proposed in any of these grants.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Carl G. Becker, M.D.

Signature [signature] Date 11/26/74

Telephone (212) 472-5984
Area Code Number Extension

Checks payable to
Cornell University Medical College

Mailing address for checks
1300 York Avenue
New York, New York 10021

Responsible officer of institution

Typed Name _____

Title _____

Signature [signature] Date 12-2-74

Telephone _____
Area Code Number Extension

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SAN RAFAEL • SANTA CLARA • SOUTH SAN FRANCISCO • SUNNYVALE • VALLEJO • WALNUT CREEK

THE PERMANENTE MEDICAL GROUP

Medical Methods Research • 3779 Piedmont Avenue • Oakland, California 94611
Morris F. Collen, M.D. • Director • (415) 645-6700

January 15, 1975

Vincent Lisante, D.M.D.
Council for Tobacco Research - USA, Inc.
110 E. 59th Street
New York, New York 10022

Dear Vince:

We very much appreciated the meeting you set up at CTR headquarters on January 9 since that gave us all a chance to exchange ideas concerning the future of our project, "Characteristics of Smokers and Nonsmokers". This was particularly useful since we were able to begin establishing priorities for future work.

One area that we all seemed to place at high priority was longitudinal studies of smokers who have had several multiphasic checkups, probably using a three-examination data base. In particular, we would place emphasis on comparisons of cigarette smokers who continue to smoke, those who quit temporarily and those who quit for as long as we can observe them. Within these latter two groups we would attempt to distinguish those ex-smokers who quit because of illness from those who quit for other reasons. Having identified these subgroups we would then focus on their initial characteristics when they were all smokers. We would first examine their age, sex and race distribution and intensity of initial smoking habit (amount, duration, inhalation). We would then look at other cardiovascular risk factors including cholesterol, blood pressure, and relative weight to see whether these differed initially before the smokers took their differing paths into the persistent and ex-smoking subgroups. Furthermore we would determine what happened to these variables subsequently, when these subjects had changed (or persisted in) their smoking status. Another area of exploration would be initial social status (as measured by educational level) and psychological traits (as measured by neuromental questionnaire items) that relate to subsequent quitting and/or persistence of smoking. These latter analyses would help determine the kinds of people who are best able to, and least able to quit smoking and remain quitters. The approximate time schedule that I would expect for this effort would be as follows.

First year: define and identify the various subgroups; conduct chart review studies concerning the reasons for stopping smoking in hopes of finding an easy method for distinguishing cessation due to illness vs. other reasons in the multiphasic data; pre-

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pare final data sets; begin analyses regarding initial demographic and smoking-intensity characteristics of the various subgroups.

Second year: complete above analyses and carry out analyses of coronary risk factors, and psychosocial data.

With a similar level of financial support we have been getting we can undertake one other major study effort during the same two-year period. The study I would favor (and I think this was generally agreed upon in our discussions) would be a case-control study of lung cancer, exploring the multiphasic examination data for factors other than smoking which are predictive of lung cancer. Stated in another way we would try to determine which smokers are most apt to get lung cancer and which are not. We would ascertain lung cancer cases who had had prior multiphasic checkups while they were free of the disease and compare them to a control group matched for smoking habit but who did not develop lung cancer. There are about 750 variables that we could look at and we have had considerable experience with similar ongoing studies of myocardial infarction, sudden cardiac death and stroke. (This approach, by the way, has a distinct advantage over the usual case-control study design, since we utilize data that were collected uniformly on the controls and on the cases before disease developed. In this way, our method resembles a cohort study).

In the proposed lung cancer study some work has already been done through a collaborative effort with Dr. John Weir, a psychologist with Dr. Petrakis's cancer study group at the University of California Medical Center in San Francisco. That study has identified 105 lung cancer cases diagnosed by 1971, and 105 smoking-matched controls who took the neuromental questionnaire at a prior multiphasic checkup. Comparison of the neuromental questionnaire response patterns is being made to determine whether psychological traits are related to the subsequent development of lung cancer as has been proposed by Kissen and others. Our proposed study would utilize these cases but we would hope to at least double the number by bringing in cases developing from 1972 through 1975 and not requiring the case to have had a neuromental questionnaire as part of his multiphasic. Incidentally, as you requested we shall invite Dr. Weir to attend the site visit to be a resource person on these matters. A copy of his curriculum vitae is enclosed.

The approximate time schedule for this lung cancer study would be as follows:

First year: ascertain additional lung cancer cases and obtain smoking-matched controls. Assemble their multiphasic data into analyzable data sets.

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Second year: data analysis -- case-control comparisons on 750 variables.

Other possibilities that were favorably considered were analyses of the neuromental questionnaire responses of persons with and without peptic ulcer, with further analyses to determine whether psychological traits might explain the smoking-peptic ulcer relationship. In addition we considered further descriptive studies of smoking habits according to occupation and birthplace. Given the projected amount of the grant, undertaking the peptic ulcer study would mean not undertaking some of the work previously described. The occupation and/or birthplace analyses might be fitted in as a secondary goal if time and resources permit, with the understanding that these were of lesser priority and that they might not be completed.

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Work in progress to be completed during the next several months. An extensive analysis of the relationship of chest pain to cigarette smoking has been completed and a manuscript was submitted for publication two weeks ago. We may have to do a small amount of additional work on this if suggested by journal reviewers. Additional analyses of smoking and urine chemistry findings have been completed. A small amount of time is needed to complete the revised manuscript. A manuscript on psychological characteristics of smokers and nonsmokers is essentially complete but some additional time is needed to clean up a few details. Partly stemming from this work another extensive study is underway to explore whether the psychological responses that distinguish smokers from nonsmokers may also account for the association of smoking with myocardial infarction. This should be completed in a few months. Over the next few months we shall also be doing some further analyses of smoking and questionnaire items about lung allergy.

Thus, in terms of a projected time schedule, I expect that the ongoing work described in the above paragraph will be completed during the first half of 1975, leaving us free to pursue the two-year program on longitudinal studies of smokers and predictors of lung cancer from July, 1975 through June, 1977.

I would propose the following agenda for our forthcoming site visit.

Greeting and Introduction	9:00 - 9:05 AM	Dr. Friedman
Overview of Kaiser-Permanente and Medical Methods Research	9:05 - 9:20	Dr. Collen
Description of data resources for proposed and other possible studies	9:20 - 9:40	Dr. Friedman
History of present grant activities and progress report	9:40 - 10:00	Dr. Friedman
Discussion	10:00 - 10:30	
Coffee break	10:30 - 10:45	
Review of Proposed Studies	10:45 - 11:15	Dr. Friedman
Discussion	11:15 - 12:00	
Lunch	12:00 - 1:00	
Tour of Multiphasic Laboratory and Health Center	1:00 - 1:30	
Discussion	1:30 -	

Members of our group who should attend in addition to Dr. Collen and myself are Dr. Seltzer, Dr. Dales and Mr. Siegelaub. Each of these persons can participate in the discussion and describe their own work in detail, if desired by the visitors. In addition, Dr. Weir would be here, and Mr. Citron, in case there is any discussion of the Twin Registry Feasibility Study.

We are, of course, quite flexible about the agenda and would be willing to modify it in any way you felt would be of help. Our purpose is to inform the visitors as fully as possible and answer any questions that might arise.

If agreeable with you we would appreciate this letter being regarded as our 6-month progress report (No. 6) for the last half of 1974, particularly the materials in the above section "Work in progress to be completed in the next several months". I might also mention that the following papers were pub-

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lished during that time, acknowledging Council for Tobacco Research - U.S.A. support.

Seltzer, C.C., Friedman, G.D., Siegelaub, A.B., and Collen, M.F.: Smoking habits and pain tolerance. Arch Environ Health 29:170-172, 1974.

Siegelaub, A.B., Friedman, G.D., Adour, K., and Seltzer, C.C.: Hearing loss in adults: relation to age, sex, exposure to loud noise, and cigarette smoking. Arch Environ Health 29:107-109, 1974.

Dales, L.G., Friedman, G.D., Siegelaub, A.B., and Seltzer, C.C.: Cigarette smoking and serum chemistry tests. J. Chron. Dis. 27: 293-307, 1974.

Seltzer, C.C., Siegelaub, A.B., Friedman, G.D., and Collen, M.F.: Differences in pulmonary function related to smoking habits and race. Amer Review of Respiratory Disease 110:598-608, 1974.

Oakes, T.W., Friedman, G.D., Seltzer, C.C., Siegelaub, A.B., and Collen, M.F.: Health services utilization by smokers and nonsmokers. Medical Care 12:958-966, 1974.

Klatsky, A.L., Friedman, G.D., and Siegelaub, A.B.: Alcohol consumption before myocardial infarction: results from the Kaiser-Permanente epidemiologic study of myocardial infarction. Annals of Internal Medicine 81:294-301, 1974.

With best regards.

Sincerely,

Gary

Gary D. Friedman, M.D.
Senior Epidemiologist

GDF:st

cc: Dr. Collen
Dr. Gardner
Dr. Hockett
Dr. Seltzer

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
AUG 27 1974

Application for Research Grant
(Use extra pages as needed)

Date: July 22, 1974

1. Principal Investigator (give title and degrees):
Gary D. Friedman, M.D., M.S. Senior Epidemiologist
Co-Investigators:
Carl C. Seltzer, Ph.D., Senior Research Associate, Harvard School of Public Health
A.B. Siegelaub, M.S., Biostatistician
Loring G. Dales, M.D., M.P.H., Epidemiologist

2. Institution & address:
Department of Medical Methods Research
Kaiser Foundation Research Institute
3779 Piedmont Avenue
Oakland, California 94611

3. Department(s) where research will be done or collaboration provided:
Medical Methods Research

4. Short title of study:
Characteristics of Smokers and Nonsmokers

5. Proposed starting date: February 1, 1975

6. Estimated time to complete: Continuation for at least one year

7. Brief description of specific research aims:

This is a request for support to continue our studies under Grant #787. Since we have already submitted information concerning items 7-13 in previous proposals and progress reports this proposal will focus on our plans and goals for the proposed renewal year.

One of our goals, of course, will be to bring to a successful conclusion, those projects and analyses that are currently in progress and are listed in the accompanying Progress Report No.5. These include studies of smoking and chest pain, differences between pipe and cigar smokers, body build in smokers and non-smokers, the relationship of blood pressure to smoking and race, and, as described, the conversion of our 1968-1973 multiphasic data to a format compatible with the 1964-1968 data we have been using. Some of these goals may be completed by the end of this year. We also plan to begin a study of the relation of smoking to onset of puberty (as judged by age at menarche and age when started shaving). While this will begin this year it will probably require additional time to complete.

When the 1964-1973 data are ready for analysis we plan to carry out longitudinal studies. One aspect of these studies would be an effort to learn more about factors affecting changes in smoking habits. For example, we shall have persons who persisted in their smoking habit over several years, persons who quit temporarily or intermittently and people who quit and did not resume smoking while under observation. It will be most interesting and worthwhile to compare the initial characteristics of these three groups while they were all smokers. Did one group smoke more than another? Were there differences in alcohol consumption, educational level or in answers to psychological questions? What are the characteristics that predict who will and who will not quite smoking? A similar analysis can be made on persons who are initially nonsmokers but who either do or do not become smokers.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

The other major area of longitudinal study that we plan to undertake is an assessment of the effect of changes in smoking habits on the characteristics we have already studied on a cross-sectional basis. We have attempted to look at changes in some of these characteristics with changes in smoking habit in our present data (e.g., in our leukocyte count and serum chemistry test papers) but we have been troubled by small numbers of subjects in certain categories and a rather short mean interval (about 1½ years) between our first and second assessment. In the nine-year data to be assembled these problems should be greatly alleviated. Of course, one of the main reasons for being so concerned about the effects of changes in habits is that this provides important additional information to help determine whether smoking is related to the characteristics we have been studying in a causal or non-causal fashion.

In summary, then, during the proposed continuation year we would complete some of our present cross-sectional studies and begin to carry out longitudinal analysis both of factors predictive of changes in smoking habits and characteristics affected by changes in smoking habits.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

Budget 2/1/75 - 1/31/76

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount Salary	Fringe	Total
Gary D. Friedman, M.D.,M.S.	20%	$ 7,620	$1,320	$ 8,940
Carl C. Seltzer, Ph.D.	20%	5,700	-	5,700
A.B. Siegelaub, M.S.	30%	7,180	1,240	8,420
Loring G. Dales, M.D.,M.P.H.	20%	5,180	900	6,080
Technical				
Programmers (2)	200%	29,200	5,050	34,250
Clerk Typist	75%	7,100	1,230	8,330
Total				71,720
Sub-Total for A				$ 71,720
B. Consumable supplies (by major categories)				
Office supplies and copying				980
Sub-Total for B				980
C. Other expenses (itemize)				
Travel by Dr. Seltzer, Boston - Oakland (3 trips)				2,100
Travel to scientific meetings (4 trips)				2,000
Data processing				20,000
Keypunching				500
Sub-Total for C				24,600
Running Total of A + B + C				97,300
D. Permanent equipment (itemize)				
None				—
Sub-Total for D				—
E. Indirect costs (15% of A+B+C)				14,600
Total request				$ 111,900

Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

6. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Sudden Cardiac Death and Myocardial Infarction	NIH-NHLI-71-2477	$ 39,650	6/16/74 - 6/14/75
Risk Factors for Stroke	NIH-NINDS-72-2319	31,310	6/14/73 - 12/31/74
Epidemiologic Study of Colon Cancer in Blacks	NIH-NCI-E-731-3213	26,415	12/1/73 - 11/30/74
Maternal X-ray Exposure	NO1 - CP - 43283	38,260	2/17/74 - 2/18/75
Multiphasic Evaluation Study	1 RO1 HS01486-01	168,700	5/1/74 - 4/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

...od that the investigator and institutional ...plying for a grant have read and accept "Statement of Policy Containing Conditions ...der Which Project Grants Are Made."

...le to

...ss for checks

Principal investigator

Typed Name Gary D. Friedman, M.D.

Signature Gary D Friedman Date July 30, 1974

Telephone 415 645-6715
Area Code Number Extension

Responsible officer of institution

Typed Name Clifford H. Keene, M.D.

Title Director, Kaiser Foundation Research Institute; President, Kaiser Foundation Hospitals

Signature Clifford H. Keene Date 8-14-74

Telephone [illegible] 271-2[illegible]

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

#1011 - LEVY

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

February 26, 1975

Grant Application No. 1011

CANCER

To: The committee comprising Drs. Gardner, Huebner, Jacobson

Subject: Jay A. Levy, M.D., University of San Francisco Cancer Research Institute
"Development of a Model In Vitro for Studying Carcinomas. Study of Possible Interplay of Endogenous Viruses and Chemicals in CoCarcinogenesis"

History

A two year plan was approved by SAB in October 1973 at a level of $40,000. Application (dated July 10, 1974) requested $60,245 and was considered as first year renewal (No. 572CR1): this was altered and considered as a new application (No. 1011) at the October 1974 SAB meeting.

The application was approved by the SAB subject to determination by the Scientific Director as to priority and budget. Also approved was a new contract proposal (MA) in the amount of $40,000 for Kouri-Levy collaboration, (see attachments) for animal holding and breeding studies to determine whether xenotropic virus/AHH segregate in mouse-breeding studies.

At a later staff meeting the Scientific Director recommended that No. 1011 and the contract be deferred until the following years budget.

Also recommended was $10,000 pro rate to Levy for continuation of his studies to March 31, 1975 (i.e. three month extension).

D.S.

DS:wg

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

[redacted]

wug file

MEMORANDUM

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH

TO : Drs. Hockett, Jacobson, Nordsiek, Sommers, Gardner

DATE: August 16, 1974

FROM : Dr. Huebner

SUBJECT: re application 572-CR1 (Jay Levy) - Dr. Gardner's comments dated August 14.

Dr. Levy is now one of the more productive and innovative investigators of RNA tumor viruses. He is already the leader in endogenous xenotropic viruses now known to be present in all cells of mice, rats, hamsters, chickens, cats, cows, pigs and old world monkeys. We now have good evidence that expressions of these viruses are frequently important in establishing certain cells in culture and also in spontaneous and chemically induced transformation. The inhibition of xenotropic virus neutralizing antibodies by MCA is an important finding and could easily be more important in chemically induced leukemias than AHH. Treatment of skin with 3MC or DMBA induces high rates of early leukemia in DBA/2's which are AHH negative (Kouri), but have lots of endogenous type C RNA viruses. In this case release of virus by immunosuppression is no doubt much more important than the AHH induction.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

WUG file

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

August 14, 1974

MEMORANDUM

TO: Drs. Hockett, Huebner, Jacobson, Nordsiek and Sommers

FROM: W. U. Gardner

SUBJECT: Application 572-CR1.
Jay A. Levy, M.D., Cancer Research Institute, University of San Francisco.
"Development of a Model System In Vitro for Studying Carcinomas. Study of Possible Interplay of Endogenous Viruses and Chemicals in Cocarcinogenesis."

This is apparently the only support that the applicant has at this time, at least none other is mentioned. This is essentially a continuation of the first year of support, the role of viruses in the malignant transformation of epithelial cells in vitro and in vivo. Levy has found xenotropic virus (X-tropic) in all cells of all tissues of NZB mice. Liver cell cultures -- NZB -- are being studied for AHH presence and capacity to transform in vitro. C57 liver cells in culture -- C57 mice have both X-tropic and ecotropic virus -- will also be studied in vitro for transformation by Mca. Attempts will be made to demonstrate C-type viruse under such conditions.

The in vivo studies will be designed to determine (1) the possible inhibition of antibodies to virus by mca, and (2) genetic factors that may influence virus expression. The genetic aspects will be studied by hybridization of NZB mice and backcrosses to mice of the C57 and 129/J strains. 129/J has antibody to X-tropic virus but has so far yielded no infectious virus. Assays for the X-tropic virus will be done using cultures of human foreskin treated with diethylaminoethy-dextran.

Other studies are planned using a melanoma growing in culture. Also attempts are being made in collaboration with Kouri to find a human X-tropic virus in culture cells treated with carcinogens and mouse liver microsomes.

The work proposed is essentially an interplay of chemical carcinogens and viruses. It has little relation to smoking except in a very broad way. He is placing more interest on epithelial cell tumors than most virologists. It will be recalled that this study started as an attempt to get cancers in cultured tracheal cells with some interesting morphological changes in the cultures.

His progress report has several tables, one listing 22 tissue culture lines from a great variety of species. He is collaborating with a number of people: Dr. Silagi on the mouse melanoma, Dr. Kouri on the genetics and AHH aspect, and others on the chemistry of the liver cell cultures.

This should be given careful consideration as to the amount of funding. He is getting away from his original objective.

W.U.G.

WUG:ek

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Source: https://www.industrydocuments.ucsf.edu/docs/mgdm0000

MAT (1)

10.14.74

Tentative budget for collaborative project with Dr. J. Levy, University of California, San Francisco Medical Center, San Francisco, California. (January 1, 1975 - December 31, 1975)

A.	Total Direct Labor (Schedule A)	$10,574.00
B.	Overhead (115% of A)	12,160.00
C.	Other direct costs (Schedule B)	8,834.00
D.	Total (A-C)	$31,568.00
E.	General and Administration (16% of D	5,051.00
F.	Total	$36,619.00
G.	Fixed Fee (10%)	4,066.00
H.	Total Costs	$40,685.00

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

MAJ (2)

Schedule A

Direct Labor

Name and Position	Time on Project	Total Hrs	$/hrs	Total
R. E. Kouri, Ph.D. Project Director	5%	96	NC	NC
T. Rude, Technician	50%	963	4.46	$4,295.
Vacancy, Lab Aide	100%	1926	3.10	5,971.
		2985		$10,266.
3% Anticipated Merit Increases				308.
		Total		$10,574.

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Source: https://www.industrydocuments.ucsf.edu/docs/mjgm0000

M43 (3).

Schedule B

Other Direct Costs

Materials		
Animals (250-129/J)	$500.00	
Feed and Bedding (total 1150 animals)	1,050.00	
Chemicals	150.00	
Total Materials		$1,700.00
Expendable Supplies		
Disposable Cages Syringes, needles, tubes	$6,100.00	
Stainless steel lids	750.00	
Total Supplies		6,850.00
Shipping		284.00
Total Other Direct Costs		$8,834.00

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

1011

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 29 1974

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐ Date: 7-10-74

1. Principal Investigator (give title and degrees):

Jay A. Levy, M.D., Assistant Clinical Professor of Medicine, Department of Medicine and Research Associate, Cancer Research Institute

2. Institution & address:

University of California
San Francisco, California 94143

3. Department(s) where research will be done or collaboration provided:

Cancer Research Institute

4. Short title of study:

Development of a model system in vitro for studying carcinomas.
Study of possible interplay of endogenous viruses and chemicals in cocarcinogenesis.

5. Proposed renewal date: January 1, 1975

6. How results to date have changed earlier specific research aims:

Our inability to grow epithelial-like cells over the past three years indicates to us that the present procedures developed offer no new aid in obtaining well differentiated epithelial cells. Instead we plan to concentrate on the NZB liver cell line in looking at a possible in vitro model for carcinogenesis. The discovery of the universality of xenotropic virus has suggested that this C-type virus may play a role in chemical carcinogenesis. Our aim therefore is to study by in vitro and in vivo systems, the activation of this virus by chemicals and to correlate these events with the serologic and pathologic response of the host.

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7. How results to date have changed earlier working hypothesis:

The results we have obtained so far have not changed any hypotheses we have had on the possible role of viruses in carcinogenesis. On the contrary, they have encouraged our studies in this field since it is clear that chemicals under certain conditions can lead to activation of viruses and perhaps dedifferentiation of normal cell function.

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

8. Any additional facilities now required? Describe briefly:

CO_2 incubator

Freezer and refrigerator

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

No

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

12. Summary progress report (append in standard form as separate document, unless recently submitted).

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested) (fringe benefits included)		
Levy, Jay A., Principal Investigator	30%	—
Technical		
Oleszko, Oksana, Staff Research Associate I	100	12,068
Ramirez, Michele, Secretary II	100	11,245
	Sub-Total for A	23,313
B. Consumable supplies (by major categories)		
Media and sera		10,500
Animals, purchase and maintenance		500
Glassware, pipettes, plastic flasks, petri dishes		10,500
CO_2 tanks and liquid nitrogen		1,000
Cell: mouse, rat, human		1,800
	Sub-Total for B	24,300
C. Other expenses (itemize)		
Travel -- domestic		800
Publication and photography expenses		800
	Sub-Total for C	1600
	Running Total of A + B + C	49,213
D. Permanent equipment (itemize)		
CO_2 incubator (NAPCO)		1,857
Freezer, cabinet, upright		1,040
Refrigerator, Lab Line		753
	Sub-Total for D	3,650
E. Indirect costs (15% of A+B+C)	E	7,382
	Total request	60,245

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Jay A. Levy, M.D.

Signature Jay A Levy Date 7-22-74

Telephone 415 (Area Code) 666-4071 (Number) (Extension)

Checks payable to

The Regents of the Univ. of Calif.

Mailing address for check:

1487 Fourth Avenue
Gifts & Endowments
University of California
San Francisco, California 94143

Responsible officer of institution

Typed Name Sue Clark

Title Gifts & Endowments - Program Coordinator

Signature Sue Clark Date 7/26/74

Telephone 415 (Area Code) 666-2047 (Number) (Extension)

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Source: https://www.industrydocuments.ucsf.edu/docs/mgdm0000

10. Proposal for continued studies

We would like to continue research centered primarily on the role xenotropic virus and chemicals may play in carcinogenesis. Both in vitro and in vivo studies are proposed.

A. In vitro experiments

In continual efforts to determine whether an epithelial cell line can be derived to provide a good in vitro model for testing chemical and viral carcinogenesis, clones of the NZB liver line will be further studied. The line has been sent to Dr. Kouri for quantitation of AHH activity. If these cells are making enzymes characteristic of liver epithelial cells and have inducible AHH activity, we shall use them for studies of chemical transformation. The cells will be treated with varying dilutions of methyl cholanthrene and observed for the formation of foci of cell alteration. Foci will be isolated and established as continuous lines. They will be inoculated into immunosuppressed mice to determine their malignant potential and their histologic cell type. Our primary aim, as stated above, is to show that mouse epithelial cells can be transformed into carcinoma cells in tissue culture. They would be very useful then for more extensive studies on the interplay of chemicals and virus in epithelial cell malignancies. Eventually, we hope such a line could be used to test the carcinogenic potential of various contaminants of our environment.

This interrelationship of C-type virus and carcinogens will be also assessed by using the C57/Black cell line (see p. 10). This epithelial-like cell line should be helpful since it cannot be transformed by standard concentrations of methyl cholanthrene and it is not making any C-type virus. Since we know C57/Black mice contain both ecotropic and X-tropic virus genomes (8,16), these cells will be treated with halogenated pyrimidines and tested for the induction of C-type virus. If a virus can be expressed after such treatment, the virus-producing cells will be exposed to methyl cholanthrene. These experiments will further substantiate whether the release of endogenous C-type virus from cells is a necessary prerequisite for carcinogenesis. In connection with these studies, the line will also be superinfected with a standard AKR type virus and then assayed for sensitivity to transformation by chemicals.

B. In vivo studies

Our initial experiments with the C57/Bl mice will be continued and should serve as a useful system for further in vivo attempts to elucidate a possible concerted role of endogenous viruses and chemicals in carcinogenesis. If we find chemical treatment results both in selective suppression of antibodies to the xenotropic virus and activation of the virus in the resulting carcinomas we have a provocative model for more detailed studies. While awaiting results in the initial experiments, we would like to begin animal studies aimed at uncovering genetic factors which may influence virus and chemical carcinogenesis. For these studies we must determine first the type of gene and/or number of genes which determine the expression of the endogenous xenotropic virus.

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We know the NZB mouse has full expression of infectious virus in all its cells even in embryonic life (8,16). By full expression we mean that any cell established in culture from that animal actively produces relatively good titers of xenotropic virus. In contrast, cells from other strains are negative

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

or yield low titers of virus and only from certain organs (e.g. spleen). The NZB strain therefore, would be used for these genetic experiments. We have found, moreover, that crosses of NZB with C57/Black or NZW mice result in progeny mice which also have full expression of infectious xenotropic virus (8,16). Virus expression in NZB mice, then, resembles the expression of ecotropic virus in AKR mice. Studies similar to those carried out to understand the genetics of the AKR virus will be followed for elucidating the genes of xenotropic virus expression (23-25).

In collaboration with Dr. Kouri, NZB mice will be crossed with 129/J mice. This strain, although possessing antibody to X-tropic virus, has not yet yielded any infectious virus (Levy, unpublished observations). The virus must then be present in very low titers. Cells from the F_1 generation, like other NZB hybrids, should produce good titers of the virus. This will be ascertained by testing tissues for virus from certain animals from this generation. Then, by crossing the (NZBx129/J)F_1 generation back to 129/J parent we can determine whether the gene for X-tropic virus expression is single or multiple. If it is single we would expect cells from fifty percent of the backcross generation to produce significant titers of X-tropic virus. If multiple genes are involved, 75% or more will maintain this ability to make virus. In conjunction with the virus studies proposed, Dr. Kouri will treat the backcross progeny with methyl cholanthrene to ascertain their susceptibility to chemical carcinogens compared to the parental line. We know both NZB and 129/J animals have a diminished capacity for induction of AHH (26,27). For these reasons higher concentrations of the chemical (300-500 µg) will be used. We would like to show that tumor induction occurs primarily in those animals which also have the genetic capability of expressing xenotropic virus.

For these studies on cocarcinogenesis, a hemisplenectomy will be performed by Dr. Kouri (without sacrificing the animal) to provide information on the AHH inductibility of the mouse under study and its capacity for spontaneous or full expression of infectious virus. The AHH assays will be conducted as already described (26,27). For virus studies, the spleens will be minced and immediately plated on human foreskin cells pretreated with diethylaminoethyl-dextran to increase the sensitivity of the assay (28). After three passages, these cells will be cocultivated with the NRK-Harvey cells. Filtered seven day supernatants from these cocultivated cultures will be assayed for focus forming activity on human and rat cell cultures. If the NZB genome for virus expressions is present we would expect titers of X-tropic pseudotype sarcoma virus to be $>10^2$/cc.

Subsequent studies would deal with selecting strains of mice with different capacities for X-tropic virus expression and aryl hydrocarbon hydroxylase activity. The following strains are being considered:

	X-tropic virus expression (15)	Aryl-hydrocarbon induction (26,27)
NZB	+	-
129/J	-	-
C57/Black	+	+
C57/Black/10	-	+

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In these experiments, the above strains will be treated with methylcholanthrene as described and the degree of tumor induction and activation

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of X-tropic virus will be determined. In those shown with good AHH activity 150 µg of MC will be used; others will receive 500 µg. Virus studies will include cultivation of the tumor resulting from MC treatment and assaying it for both ecotropic and xenotropic virus production by standard techniques (8,16). Moreover, during all these studies, serum samples will be collected for determination of neutralizing antibody titers to ecotropic and xenotropic viruses.

C. Melanoma cells

We would like to continue studies on this interesting epithelial cell carcinoma. We want to determine whether one particular event in melanoma cells results in both virus activation and dedifferentiation as reflected by the decrease in pigment formation. Moreover, we would like to see if viruses, particularly the xenotropic virus, can be induced in this line by methyl cholanthrene or other hydrocarbons as well as halogenated pyrimidines and/or protein inhibitors (29). Finally, we would like to note if resumption of pigment formation (which occurs with certain cells of this cell line after IUDR has been removed) correlates with a decrease in virus production or more specifically in a selective change in the type of virus progeny being made. Finally, the loss of malignancy with virus activation will be evaluated. These studies are aimed at examining conditions by which differentiation, carcinogenesis, and virus expression are interrelated. Moreover, mechanisms by which malignant changes are maintained by the cell can be investigated.

D. Human xenotropic virus

Dr. Kouri is beginning a study directed at determining if human cells can be transformed by chemical hydrocarbons when microsomal fractions of mouse liver homogenates are used as a source of the AHH enzymes needed for production of the active chemical factors. Any transformed cells will be sent to us for evaluation for possible human xenotropic C-type virus. These studies will include:

1. Examination by electron microscopy.

2. Measurement of reverse transcriptase.

3. Cocultivation of the cells with a variety of animal cells which we are cultivating in our laboratory. A list of some of these cells and their growth capabilities is given in Table VI.

Significance

Theories on the role of viruses in cancer have suggested that a genetic message for a virogene and oncogene exist in all living cells (9). The discovery of the xenotropic virus has provided support of the virogene hypothesis, but the connection of this genetic message to oncogenes has not been established. Chemical carcinogenesis appears to be different from viral oncogenesis since chemicals require probably two steps for ultimate tumor induction (30). The experiments outlined in this proposal are aimed at elucidating the basic interplay between two factors, chemicals and viruses (particularly xenotropic viruses), in the possible production of carcinomas. They are designed to demonstrate that oncogenes may require

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

other determinants for expression than virogenes even if both are part of the same genetic molecule. The establishment of an epithelial line from the NZB liver, if confirmed by subsequent studies, represents the first true epithelial type cell line obtained from mice and promises to be very useful for testing various factors, particularly chemicals, for their carcinoma-inducing properties.

The role of the xenotropic virus has not been demonstrated. The cause of carcinogenesis (chemical or spontaneous) has not been determined and C-type viruses capable of producing epithelial cell tumors have not been isolated. It seems logical to us that viruses that are clearly endogenous to all members of a species may be responsible for common tumors which occur probably by interaction with environmental stimuli or common chemical contaminants. Since epithelial tumors represent the majority of cancers present in man, further studies on epithelial cell carcinogenesis and xenotropic viruses is important. The unraveling of this mystery may indeed bring us closer to the basic understanding of chemical carcinogenesis and the means by which we may prevent it.

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

12. Semi-Annual Progress Report

I. INTRODUCTION

Eighty percent of human cancers are carcinomas, and the great number of these occur in the lung. It is apparent to us that in vivo and in vitro systems directed at studying the induction of carcinogenesis in normal epithelial cells are important in trying to understand the variables which could be responsible for carcinoma production. Moreover, the investigation of carcinoma cells established in culture may help us understand the factors responsible for maintaining the malignant changes. For these reasons, our research has dealt primarily with three related topics.

A. Development of a model in vitro system for studying carcinoma induction.

B. Studies on the relationship of xenotropic viruses to chemical carcinogenesis.

C. Studies of carcinoma cells in vitro.

A. Development of a model in vitro system for studying carcinomas.

1. Background and Recent Results

Our initial research dealt with attempts to develop a model system in tissue culture for studying epithelial cell transformation. We chose the mouse system not only because of the readily available inbred strains, but also because it is a mammalian cell system which has endogenous RNA viruses that are well-understood and characterized. Tissue culture techniques would permit rapid methods for studying the possible concerted role of viruses and chemicals in epithelial cell malignancies. Moreover, once an epithelial line is established, various contaminants in our atmosphere can be checked for their carcinogenic potential.

In the course of this experimentation we learned that cell morphology in tissue culture is not an accurate method of distinguishing cell types (see Reports to the Council 1972, 1973). This conclusion is supported by the results of Sanford, et al. and others (1-3) who have noted that epithelial cells can change into fibroblast-like cells and vice versa. Based on our results with cultivated epithelial-like cells (see below) we have realized that the most accurate method for determining whether true epithelial cells have been cultured, and have been transformed, is the in vivo production of carcinomas in specific animal hosts. For this reason, our ultimate determination of epithelial cell histology is the induction of carcinomas in immunosuppressed mice.

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We initially attempted to cultivate epithelial cells from mouse tracheas or skin by a variety of different techniques. All these attempts yielded islands of epithelial-like cells in culture but after a period of one to two weeks the surviving epithelial-like cells either spontaneously changed or they were replaced by fibroblast-like cells which grew rapidly to confluency. On occasion we observed that some islands grew and maintained characteristics of epithelial cells. Their cell division occurred only at the periphery and confluency required a period of one or two months. Petri dishes then became

Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000

filled with cells morphologically resembling epithelial cells. The same cells, however, when transformed by murine sarcoma viruses, or by the combined activity of chemicals and murine leukemia virus, only induced fibrosarcomas not carcinomas in immunosuppressed mice (Experiments conducted in collaboration with Dr. Paul Arnstein, Berkeley).

Our recent studies have dealt with established cell lines which have epithelial-like cell morphology.

a. NZB mouse epithelial-like cell line (C-1,S-2)

We have derived an epithelial-like cell line from NZB embryo cells. Since these cells produce xenotropic murine leukemia virus (see p. 11) they seemed ideal for studying directly the interaction of chemicals with endogenous C-type viruses. The line was tested for aryl hydrocarbon hydroxylase (AHH) by Dr. Richard Kouri (MBA, Bethesda) and was found to have low levels of the enzyme. This cell line (C-1,S-2) when treated with a small amount of methyl cholanthrene (MC) or benzo(a)pyrene produced foci of cell transformation which were easily recognized in the cell monolayer (see Fig. 4,5 of Annual Report, 1973). The cells therefore seemed to provide a good system for measuring potential carcinogenic compounds in vitro. To enhance the susceptibility of the cells to chemical transformation, pretreatment with non-carcinogenic hydrocarbons was tried. The results indicated, however, that pretreatment did not significantly increase the amount of focus formation induced by small amounts of MC (0.1 ug/cc). Moreover, foci isolated from this cell line and established in culture have produced only sarcomas in mice. These results indicated that either the cells when transformed reverted to a fibroblast morphology, or they originated from fibroblast cells with epithelial cell morphology. Recently these epithelial-like cells when maintained up to passage 9-12 spontaneously developed foci which did not appear to be any different from those induced by chemical alone, nor did they produce any different type of tumor in inoculated immunosuppressed NIH Swiss mice (Arnstein, personal communication). Such results, though telescoped in fewer passages with the NZB line, resembled the data published by Freeman, et al. (4-6). These authors found that the same rat cell line which could be transformed by the combined treatment with chemical and virus developed spontaneous foci of similar cell alteration after numerous passages. Since spontaneous foci occurred at early passages in this NZB cell line and the transformed cells only produced sarcomas, we decided to abandon the use of this cell line and attempt similar experiments with other epithelial-like cell lines established in culture.

2. C57/Black mouse epithelial-like cell line

We have derived from the peritoneum of a C57/Bl mouse, an epithelial-like cell culture, which is not releasing any xenotropic virus. When exposed to various concentrations of methyl cholanthrene, higher levels of the chemical were needed for toxicity (5 ug/cc) than seen with the NZB embryo cell line, and no transformation occurred. Cells remaining in the cultures which received toxic doses of MC were maintained with the hope that transformation would be noted. However, these surviving cells resumed their epithelial morphology and no transformation occurred after 10 passages. This line will be further evaluated for its ability to be transformed after superinfection with standard MLV or after induction of X-tropic virus (see p. 5).

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3. A liver cell line from an NZB mouse has been established. It has an epithelial-like cell morphology; it is discussed in the next section (see below).

B. The relationship of xenotropic virus to chemical carcinogenesis

1. Background

Four years ago in a study of NZB mice we were able to demonstrate the presence of a C-type RNA tumor virus which has an unusual tropism (7). Instead of being infectious for cells from its own animal species, it could only be propagated in cells from animals of different species; for this reason it has been called a xenotropic (X-tropic) virus (8). We have been able to isolate this virus from several mouse species and believe it is a common endogenous virus of mice (Table 1). Moreover, sera from most if not all mouse strains, neutralize the virus at titers of at least 1:100. NZB mice and their hybrids usually have titers of 1:1000 and occasionally titers of 1:10,000 have been detected (Levy, manuscript in preparation) (Table II).

The role of this mouse xenotropic virus has not yet been determined. Because many chemically induced tumors do not yield any standard mouse C-type virus and yet have CF antigen, we decided that these tumors should be examined for the presence of xenotropic virus. There was the possibility as suggested by Huebner, et al. (9-12) that the interaction of chemical with endogenous virus could result in carcinogenesis. Both in vitro and in vivo studies have been begun aimed at elucidating this theory.

a. In vitro studies

We believe the most efficient method of looking at the induction of carcinoma by the interaction of chemical and virus is by in vitro testing. As noted above, we have attempted to grow cells from epithelial tissues and test their susceptibility to chemical and/or virus transformation. We were especially interested in developing a line from NZB mice since these cells would be ideal for studying direct interactions of chemical and endogenous C-type virus.

Our initial efforts to establish an epithelial cell line from NZB cultures resulted in the establishment of the C-1,S-2 clonal cell line. Although epithelial-looking morphologically, this line, after transformation, only produces sarcomas in mice and so its value as an in vitro model of carcinomas was diminished.

From further studies on other lines derived from NZB mice and their hybrids a line from an NZB liver culture has been established. These cells grow in a very unusual pattern. Instead of assuming whorls or parallel accumulations of fibroblast-like cells, these "liver" cells maintain a pavement-like appearance and grow to confluency without significant piling up (Fig. 1). The most interesting feature of these cells is their pattern of growth which leaves areas free of cells scattered throughout the monolayer (see Fig. 2). The picture suggests liver parenchymal cells allowing spaces for the in-growth of blood vessels or bile canaliculi.

One-cell clones from the established line (3105) continue to maintain this unusual morphologic characteristic. Using standard techniques (7,8) we have determined that this line, as have all cells from NZB mice, produces xenotropic C-type virus. The cells were cocultivated with the NRK-Harvey

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cell line, a non-virus producing rat cell line transformed by the Harvey sarcoma virus (13). After seven days, the supernatant fluids from the cocultivated cultures were tested for focus activity in human cells and significant amounts of pseudotype sarcoma virus were detected. The line, therefore, if epithelial would permit us to conduct in vitro studies on the possible interplay of endogenous C-type viruses and chemicals in carcinogenesis. Before initiating any extensive studies on this line, we did not want to be faced with the same problem presented by the C-1,S-2 line, i.e. epithelial-looking cells which may be fibroblasts. The "liver" line offered some advantage over the other epithelial-like cells since if epithelial, it should be making liver enzymes.

The cells have been examined by Dr. Urs Meyer in Dr. R. Schmidt's laboratory for the presence of the liver enzyme, delta-levulenic acid synthetase but were negative. Such a result, however, is not unusual for liver cells established in culture from other species (Meyer, personal communication). They have been tested by Dr. Ulrich Gehring in Dr. Tomkins' laboratory for the presence of tyrosine aminotransferase enzyme. The first set of experiments have been very encouraging. Enzyme activity, though at a low level, was detected in the cultures and this activity was increased three-fold by treating with dexamethasone. The cells, therefore, behave similarly to the rat hepatoma cell line (HTC) which Tomkins, et al. use in dexamethazone induction studies (14). Dr. Gehring believes the data suggest 3105 is a mouse liver line and is doing further studies on it to confirm these initial results. The line is also being evaluated for the presence of the liver enzymes, glucose-6-phosphatase and B-hydroxy-B-methyl glutaryl coenzyme A reductase (15) by Dr. M. Siperstein of the Department of Medicine.

If these tests confirm the fact that the cells are liver parenchymal cells, we shall proceed with standard techniques for studying chemical transformation in vitro. This line has been inoculated into antithymocyte serum (ATS) treated NIH Swiss mice and nude mice. No tumors have appeared after 3 months (Arnstein, personal communication). This suggests that the line has not spontaneously transformed.

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In vivo studies

In collaboration with Dr. Richard Kouri (MBA, Bethesda) a prospective study has been begun of chemical carcinogenesis in inbred strains of mice. The C57/Black strain was chosen for the first experiment since it has xenotropic virus as well as mouse-tropic (ecotropic) virus (8,16) so we could assess to some extent the role of both these viruses in carcinogenesis. We know that sera from C57/Black mice generally can neutralize xenotropic pseudotype sarcoma virus at titers of 1:100-1:300 (Table II); sometimes titers as high as 1:1000 are observed. We are interested in determining whether treatment of C57/Black mice with methyl cholanthrene decreases their antibody titer to xenotropic virus and permits the emergence of endogenous X-tropic virus and the ultimate production of carcinomas. For our initial study, 25 C57/Bl mice were treated with MC diluted in triocane. Twenty-five control mice received only the vehicle. Blood samples were taken prior to treatment and will be taken every three weeks after treatment until the development of carcinomas. In some animals these carcinomas will be removed and the animals maintained for another 3 months. In preliminary studies on four C57/Black mice already carrying chemically induced tumors the xenotropic virus was demonstrated in two out of the four cases (Table III). In two tumors, a B-tropic ecotropic virus was isolated. This high frequency of recovery of B-tropic virus was surprising and may reflect the increased sensitivity of

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our assays which include passage of indicator mouse and human cells for three weeks before testing them for ecotropic and/or xenotropic virus.

We are now three months into this study of chemically induced tumors and the results on the mouse sera selected from the 50 mice in this study are given in Table IV. They indicate that antibody titers to the virus are present before treatment with the chemical and range between 1:100 and 1:1000. In a preliminary survey of some sera obtained 2-7 weeks after MC treatment, the titer of antibody to the X-tropic virus was found to be diminished. We shall compare these results with the control and vehicle-treated mice to determine if they are significant. We are encouraged, however, by these first observations since antibody studies in our laboratory with other C57/Black mice do not show a drop in titer particularly to levels less than 1:10. These results suggest some relationship may exist between chemical exposure, virus activation without virus neutralizing antibody response, and the ultimate development of malignancy.

C. Studies of carcinoma cells in vitro: C57/Bl melanoma cell line

We are collaborating with Dr. Selma Silagi (New York) in studies directed at determining factors which induce or maintain carcinogenesis. Dr. Silagi has derived from C57/Bl mice a continuous line of malignant melanoma cells which produce pigment in tissue culture (17). After the addition of halogenated pyrimidines, however, pigment formation ceases, the cells begin to produce C-type virus, and they become less malignant for animal hosts (18,19). We have confirmed the results on virus activation in our laboratory. Initially experiments were conducted in which melanoma cells were cocultivated for three weeks in the presence of NIH Swiss mouse embryo, BALB/c, mouse embryo and human foreskin cells. Following these procedures, the mouse cells were cocultivated with XC cells to detect mouse-tropic (ecotropic) virus and the human cells were cocultivated with NRK-Harvey cells to detect the presence of xenotropic virus.

In these studies no virus could be detected in the melanoma cell line itself, even after long-term cultivation with the indicator lines. Nevertheless, within two days after 100 ug/cc of iododeoxyuridine (IUDR) was added to the culture, pigment formation decreased and virus production could be detected. The viruses thus far isolated have been of two types, N-tropic and B-tropic (20). (Table V) No X-tropic virus has yet been detected. The recovery of B-tropic virus is unusual since most latent viruses activated in tissue culture from B-type cells are N-tropic (21,22). Certain lines of these melanoma cells have spontaneously begun to release B-tropic virus and they produce less pigment than the non-virus producing melanoma cells. It appears then that dedifferentiation or decreased pigment formation occurs at the time of C-type virus production. These cells provide an excellent opportunity to look at the interplay of differentiation, virus activation and epithelial cell carcinogenesis.

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TABLE I

RECOVERY OF XENOTROPIC VIRUSES FROM VARIOUS MOUSE STRAINS

MOUSE STRAINS	POSITIVE/TOTAL	RANGE IN VIRUS TITER (FFU)/cc
NZB	42/42	1-1000
(NZBxNZW)F_1	8/8	1-1000
(NZBxC57/Bl)F_1	4/4	1-200
NZW	2/2	1-1000
C57/Black	5/8	1-1000
NIH Swiss	12/16	1-1000
C57/Leaden	1/1	1-50
BALB/c	1/2	1-20
Nude	2/2	1-20
C57/Black 10 (Snell)	2/2	1-20
C57/Black 10 (58N)	2/2	1-20
SJL/J	0/5	-
SWR	0/2	-
129/J	0/2	-
A/J	0/2	-

[1]Spleen, thymus, and kidney from each adult animal was assayed for infectious xenotropic virus by standard technique. Recovery of the virus from one of these organs is registered as a positive.

[2]Number of infectious pseudotype sarcoma virus particles produced after cocultivation of cells from these mice with non-virus producing MSV-transformed rat cells ( ). The titer was determined by focus forming activity (FFU) in human or rat cells. The number gives a general estimate of the amount of infectious xenotropic virus being produced by the cultured cells.

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TABLE II

NEUTRALIZATION OF XENOTROPIC VIRUS BY SERA FROM VARIOUS MOUSE STRAINS

STRAIN	NUMBER OF ANIMALS TESTED	AVERAGE TITER[1]
NZB	8	1000-10,000
(NZBxNZW)F_1	8	1000-4000
NZW	3	100-200
C57/Black	15	100-200
NIH Swiss	23	10-100
C57/Leaden	9	100-200
SJL/J	3	100-200

1. Neutralization titer was determined by the neutralization of the focus forming activity of xenotropic pseudotype sarcoma virus.

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TABLE III

RECOVERY OF MLV FROM CHEMICALLY-INDUCED TUMORS IN C57/BLACK MICE

	ANIMAL	X-TROPIC	E-TROPIC
1.	TUMOR	+	0
	SPLEEN	0	0
2.	TUMOR	0	0
	SPLEEN	+	0
3.	TUMOR	0	0
	SPLEEN	+	+
4.	TUMOR	+	+
	SPLEEN	+	+

[1]Four C57/Black mice were inoculated subcutaneously with 3-methyl-cholanthrene. Tumors developed after 3 months at the site of inoculation. These animals were sacrificed and their tumors and spleens were cultivated and tested for the presence of xenotropic (X-tropic) and ecotropic (E-tropic) murine leukemia viruses (MLV).

[2]All ecotropic isolates were of the B-tropic subgroup of MLV.

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TABLE IV

EFFECT OF METHYL-CHOLANTHRENE (MC) ON SERUM ANTIBODIES TO THE MURINE XENOTROPIC VIRUS[1]

Animal No.	Before Treatment	2 Weeks After MC Treatment	4 Weeks After MC Treatment	7 Weeks After MC Treatment
472	100	-	100	10
473	100	-	-	
474	1000	10	10	10
475	1000	-	-	
476	1000	-	<10	<10
477	100	-	-	
478	1000	-	-	
482	-	10	10	<10
484	-	10	10	<10
487	-	-	10	10
494	-	-	10	-
495	-	-	10	-
496	-	-	100	<10

[1]Numbers represent the serum dilution which gave at least 66% neutralization of focus formation by NZB pseudotype sarcoma virus.

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TABLE V

RECOVERY OF C-TYPE VIRUSES FROM CULTURED C57/BLACK MELANOMA CELLS

Cells	Treatment	Virus Recovered
Melanoma	0	0
	IUDR alone	B-tropic AKR type
	IUDR + BALB-ME	B-tropic AKR type
	IUDR + NIH-ME	N-tropic AKR type
	IUDR + HuF	0

Cells were exposed to iododeoxyuridine (IUDR), 100 µg/cc for 36 h. Cultures were then washed and refed with maintenance media. To certain treated cultures, BALB/c or NIH Swiss mouse embryo (ME) or human foreskin (HuF) cells were added. After 7 days, the fluids were collected and titered on NIH-ME and BALB/c ME. The cultures receiving HuF cells were passed three times and then cocultivated with NRK-Harvey cells. The 7-day supernatant from that culture was tested for X-tropic pseudotype sarcoma virus by focus formation assay on human cells.

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TABLE VI

ANIMAL CELL LINES PRESENTLY CULTIVATED IN THE LABORATORY

Lines Available	Growth Characteristics
Mammalian:	
Anteater	Large, fat fibroblast, very granular, moderate growth rate.
Bat lung	Small cells, epithelioid, very rapid growth.
Bear lung	Large fibroblast cell, rapid growth rate.
Bovine embryo kidney	Fibroblast cell, moderate growth rate.
Deer kidney	Fibroblast, fairly rapid growth.
Dog kidney	Epithelioid, very rapid growth (similar to bat lung.
Gazelle lung	Fibroblastic cells, fairly rapid growth rate.
Chinese hamster embryo	Fibroblastic cells, moderate growth rate.
Chinese hamster thymus	Fibroblastic cells, moderate growth rate.
Syrian hamster embryo	Fibroblastic cells, moderate growth rate.
Lion lymph node	Large fibroblast, moderate growth rate.
Embryonic rhesus monkey heart	Fibroblastic cells, moderate growth rate.
African water mongoose	Large fibroblast, moderate growth rate.
Peccary kidney	Very vacuolated granular epithelial-like cells; rapid growth.
Racoon uterus	Fibroblastic cell; moderate growth rate.
Avian:	
White Longhorn chicken	Fibroblastic cells, moderate growth rate.
Peking duck	Fibroblastic cells, moderate growth rate.
Red-necked pheasant	Fibroblastic cells, moderate growth rate.
Golden-necked pheasant	Fibroblastic cells, moderate growth rate.
Quail	Fibroblastic cells, moderate growth rate.
Insect:	
Mosquito Chao A	Epithelial, vacuolated cells, very fast growth at room temperature.
Aedes albopictus	Epithelial, vacuolated cells; very fast growth at room temperature.

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REFERENCES

1. Sanford, K.K., Westfall, B.B., Chu, E.H.Y., Kuff, E., Cavalsky, A.B., Dupree, L.T., Hobbs, G.L. and Carle, W.R. Alterations in morphology, arginase and β-glucuronidase within a clone of mouse tumor cells in vitro. J. Natl. Cancer Inst. 26: 1193-1219, 1961.

2. Sanford, K.K., Likely, G.D. and Earle, W.R. The development of variations in transplantability and morphology within a clone of mouse fibroblasts transformed to sarcoma-producing cells in vitro. J. Natl. Cancer Inst. 15: 215-232, 1954.

3. Yuspa, S., Morgan, D.L. and Levy, J.A. In vitro cultivation of a chemically induced epidermal carcinoma: Establishment of three cell lines and isolation of murine leukemia virus. J. Natl. Cancer Inst. 50: 1561-1570, 1973.

4. Freeman, A.E., Price, P.J., Igel, H.J., Young, J.C., Maryok, J.M. and Huebner, R.J. Morphological transformation of rat embryo cells induced by diethylnitrosamine and murine leukemia viruses. J. Natl. Cancer Inst. 44: 65-78, 1970.

5. Freeman, A.E., Kelloff, G.J., Gilden, R.V., Lane, W.T., Swain, A.P. and Huebner, R.J. Activation and isolation of hamster-specific C-type RNA viruses from tumors induced by cell cultures transformed by chemical carcinogens. Proc. Natl. Acad. Sci. USA 68: 2386-2390, 1971.

6. Freeman, A.E., Gilden, R.V., Vernon, M.L., Wolford, R.G., Hugunin, P.E. and Huebner, R.J. 5-Bromo-2'-deoxyuridine potentiation of transformation of rat-embryo cells induced in vitro by 3-methylcholanthrene: Induction of rat leukemia virus gs antigen in transformed cells. Proc. Nat. Acad. Sci. USA 70: 2415-2419, 1973.

7. Levy, J.A. and Pincus, T. Demonstration of biological activity of a murine leukemia virus of New Zealand Black mice. Science 170: 326-327, 1970.

8. Levy, J.A. Xenotropic viruses: murine leukemia viruses associated with NIH Swiss, NZB, and other mouse strains. Science 182: 1151-1153, 1973.

9. Huebner, R.J. and Todaro, G.J. Oncogenes of RNA tumor viruses as determinants of cancer. Proc. Natl. Acad. Sci. 64: 1087-1094, 1959.

10. Huebner, R.J., Todaro, G.J., Sarma, P., Hartley, J.W., Freeman, A.E., Peters, R.L., Whitmire, C.E., Meier, H. and Gilden, R.V. "Switched off" vertically transmitted C-type RNA tumor viruses as determinants of spontaneous and induced cancer: a new hypothesis of viral carcinogenesis. In: Defectiveness rescue and stimulation of oncogenic viruses. 2nd International Symp. on Tumor Viruses, No. 183. Paris: 1970, pp. 33-57.

11. Huebner, R.J., Kelloff, G.J., Sarma, P.S., Lane, W.T. and Turner, H.C., Gilden, R.V., Oroszlan, S., Meier, H., Myers, D.D. and Peters, R.L. Group-specific antigen expression during embryogenesis of the genome of the C-type RNA tumor virus: Implications for ontogenesis and oncogenesis. Proc. Nat. Acad. Sci. 67: 366-376, 1970.

10035465 93

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12. Huebner, R.J., Freeman, A.E., Whitmire, C.E., Price, P.J., Rhim, J.S., Kelloff, G.J., Gilden, R.V. and Meier, H. Endogenous and exogenous RNA tumor virus genomes in chemical carcinogenesis. In Environment and Cancer, A Collection of Papers Presented at the Twenty-Fourth Annual Symposium on Fundamental Cancer Research, 1971, Baltimore, The Williams & Wilkins Co., 1972, pp. 318-345.

13. Levy, J.A. Demonstration of differences in murine sarcoma virus foci formed in mouse and rat cells under a soft agar overlay. J. Natl. Cancer Inst. 46: 1001-1007, 1971.

14. Tomkins, G.M., Levinson, B.B., Baxter, J.D. and Dethlefsen, L. Further evidence for post-transcriptional control of inducible tyrosine aminotransferase synthesis in cultured hepatoma cells. Nature New Biol. 239: 9-14, 1972.

15. Brown, M.S., Dana, S.E., Dietschy, J.M. and Siperstein, M.D. 3-hydroxy-3-methylglutaryl coenzyme A reductase. J. Biol. Chem. 248: 4731-4738, 1973.

16. Levy, J.A. Autoimmunity and neoplasia: the possible role of C-type viruses. Am. J. Clin. Path. In press.

17. Silagi, S. Control of pigment production in melanoma in vitro: evocation and maintenance. J. Cell Biol. 43: 263-274, 1969.

18. Silagi, S. and Bruce, S.A. Suppression of malignancy and differentiation in melanotic melanoma cells. Proc. Nat. Acad. Sci. 66: 72-78, 1970.

19. Silagi, S., Beju, D., Wrathall, J. and DeHarven, E. Tumorigenicity, immunogenicity, and virus production in mouse melanoma cells treated with 5-bromodeoxyuridine. Proc. Nat. Acad. Sci. USA 69: 3443-3447, 1972.

20. Hartley, J.W., Rowe, W.P. and Huebner, R.J. Host-range restrictions of murine leukemia viruses in mouse embryo cell cultures. J. Virol. 5: 221-225, 1970.

21. Aaronson, S.A., Todaro, G.J., and Scolnick, E.M. Induction of murine C-type viruses from clonal lines of virus-free BALB/3T3 cells. Science 174: 157-159, 1971.

22. Todaro, G.J. "Spontaneous" release of type C viruses from clonal lines of "spontaneously" transformed BALB/3T3 cells. Nature New Biol. 240: 157-160, 1972.

23. Rowe, W.P. Studies of genetic transmission of murine leukemia virus by AKR mice. I. Crosses with Fv-1^n strains of mice. J. Exper. Med. 136: 1272-1285, 1972.

24. Rowe, W.P. and Hartley, J.W. Studies of genetic transmission of murine leukemia virus by AKR mice. II. Crosses with Fv-1^b strains of mice. J. Exper. Med. 136: 1286-1301, 1972.

25. Meier, H., Taylor, B.A., Cherry, M. and Huebner, R.J. Host-gene control of type C RNA tumor virus expression and tumorigenesis in inbred mice. Proc. Nat. Acad. Sci. (Wash.) 70: 1450-1455, 1973.

10035465 94

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26. Kouri, R.E., Ratrie, H. and Whitmire, C.E. Genetic control of susceptibility to 3-methylcholanthrene-induced subcutaneous sarcomas. Int. J. Cancer 13: 714-720, 1974.

27. Gielen, J.E., Goujon, F.M. and Nebert, D.W. Genetic regulation of aryl hydrocarbon hydroxylase induction. II. Simple Mendelian expression in mouse tissues in vivo. J. Biol. Chem. 247: 1125-1137, 1972.

28. Duc-Nguyen, H. Enhancing effect of diethylaminoethyl-dextran on the focus-forming titer of a murine sarcoma virus (Harvey strain). J. Virol. 2: 643-644, 1968.

29. Aaronson, S.A. and Dunn, C.Y. High-frequency C-type virus induction by inhibitors of protein synthesis. Science 183: 422-423, 1974.

30. Berenblum, I. A speculative review: The probable nature of promoting action and its significance in the understanding of the mechanism of carcinogenesis. Cancer Res. 14: 471-477, 1954.

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LEGENDS TO FIGURES

Figure 1. Culture of epithelial-like cells derived from an NZB liver. Note the cells pavement-like appearance of the rectangular-shaped cells. X100.

Figure 2. Culture of epithelial-like cells derived from an NZB liver. Note the spaces free of cells. It gives the appearance of liver parenchymal cells allowing room for the ingrowth of blood vessels or bile canaliculi. X40.

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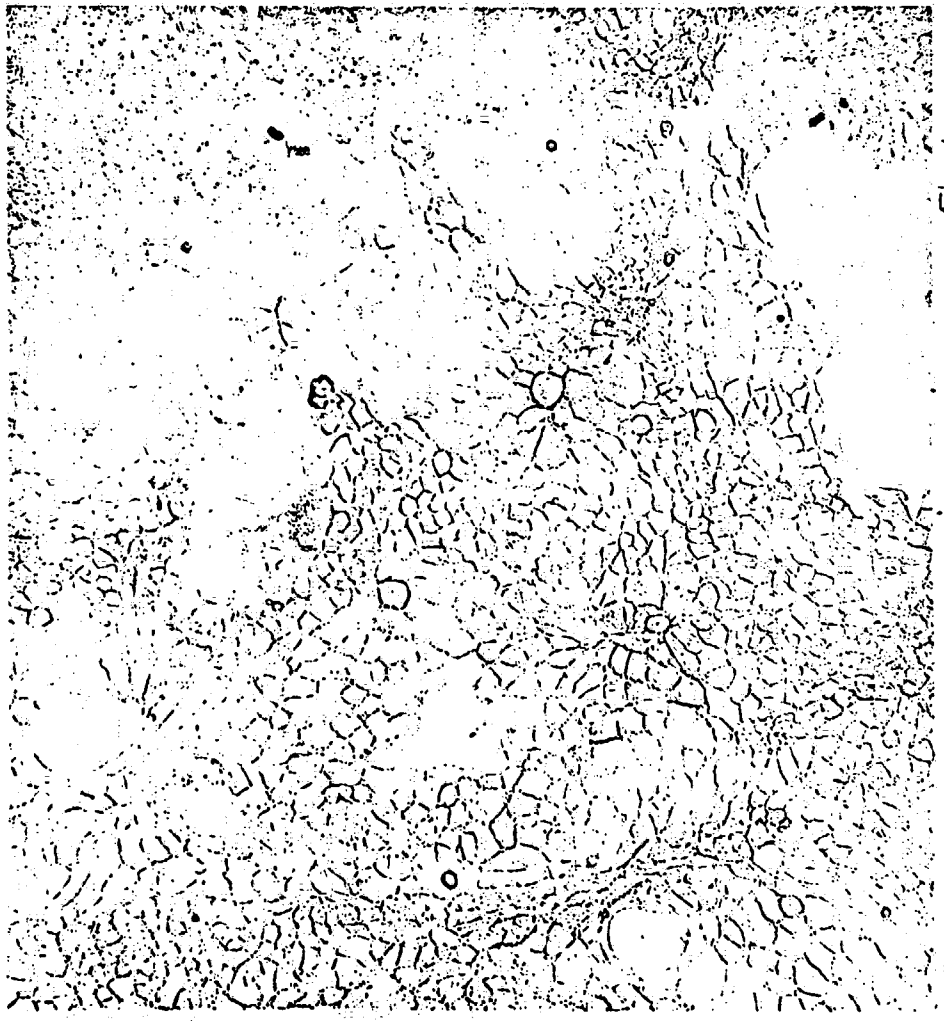

Fig 1

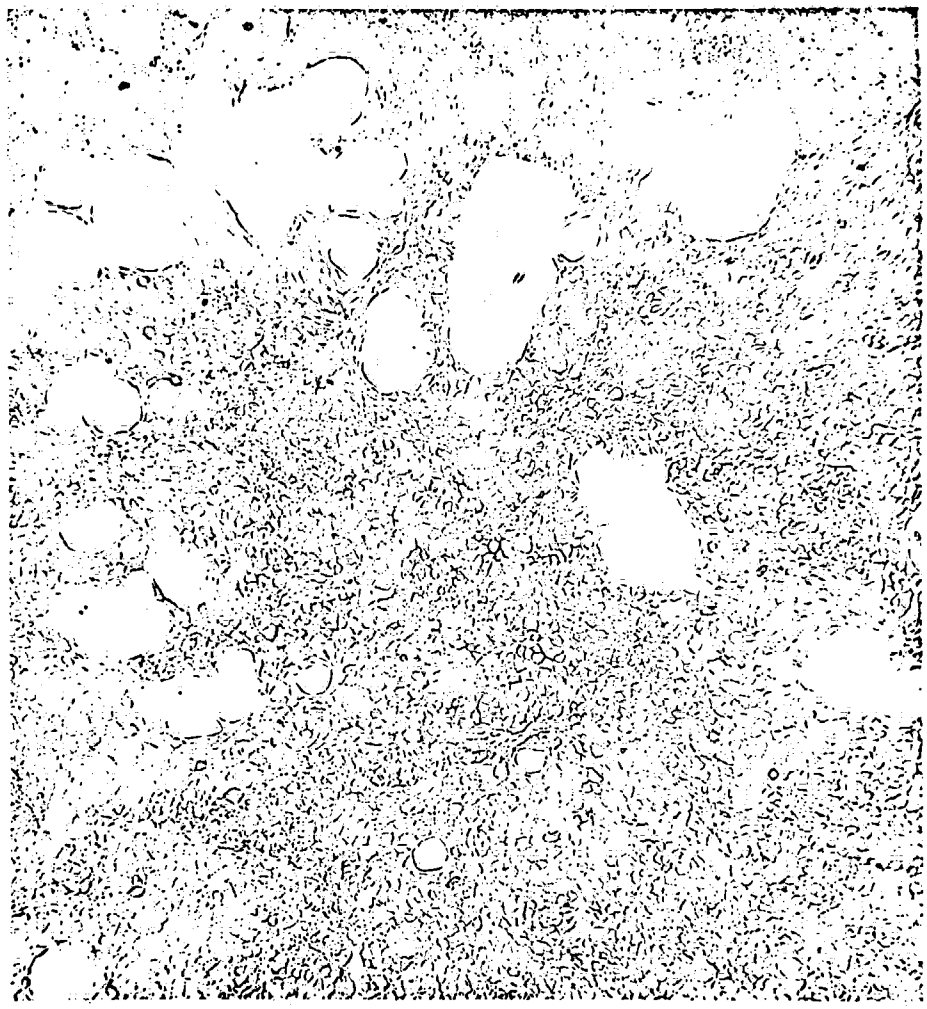

Fig 2

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Copy to Dr. Hoefe [illegible]

LENOX HILL HOSPITAL

100 EAST 77TH STREET / NEW YORK, N. Y. 10021

Founded 1857

December 19, 1974

Dr. David Stone
The Council for Tobacco Research - USA, Inc.
110 East 59th. Street
New York, New York 10022

Dear Dr. Stone:

Recently, we discussed certain criticisms regarding my research proposal #996. These criticisms focussed chiefly about the total number of patients to be studied; the composition of the patient group with regard to normal controls; and the necessity for meaningful epidemiological data. I have carefully considered them and feel that certain important modifications would be in order.

The requirement for study of greater numbers of cases, say eighty over a period of three years is the most important consideration. This will require (i) a full-time technical assistant (ii) an analytical electron microscope at Lenox Hill Hospital (iii) considerably more of my professional time. The reasons for these changes are as follows: Processing of greater numbers of new cases with fixation, embedding, mucous membrane orientation, thick-sectioning, thin-sectioning, staining, carbon-filming and subsequent photographic printing obviously will require the entire attention of a qualified technical assistant. In order to insure the highest quality results, I feel that I must oversee the microanalysis. It is clear to me that unless I am present when this is done, exact cellular localization will not be accomplished. Since treble the number of cases than originally proposed must be studied, I cannot afford the time for so many trips to the Massachusetts Application Laboratory and thus the equipment has to be at Lenox Hill Hospital. With regard to control cases, a minimum number can be agreed upon and will be obtained from the Medical Examiner's office as accident or homicide victims. I have enclosed a copy of a sample epidemiological questionaire. I am quite willing to discuss modifications of the questionaire if desired so as to make the accumulated data as meaningful as possible.

The revised costs of the proposal then are as follows: analytical electron microscope with x-ray energy detector, side entry goniometer stage and high resolution scanning transmission attachment - $112,700.00; annual budget, first year - $30,557.80; second year - $30, 557.80, third year - $31,132.80; total costs over three years $204,948.40.

If this is done, meaningful data will be obtained to aid in our understanding of lung diseases associated with inorganic materials.

Sincerely yours,

JAT:pf

John A. Terzakis, M.D.

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NAME _____

PRESENT ADDRESS _____

AGE _____ SEX _____

ADDRESSES IN PAST THIRTY (30) YEARS

YEARS (APPROX.)	CITY	STATE

INDUSTRIAL EXPOSURE TO DUSTS, GASES, OR TOXIC FUMES EITHER AS EMPLOYEE OR IN NEIGHBORHOOD AIR:

TYPE, IF KNOW, OR COMPANY NAME	DURATION OF EXPOSURE

HAVE YOU EVER HAD ANY OF THE FOLLOWING:

BRONCHIAL ASTHMA
CHRONIC BRONCHITIS
EMPHYSEMA
TUBERCULOSIS
LUNG CANCER

FAMILY HISTORY INCLUDING LUNG DISEASE AND CANCER

MOTHER

FATHER

SISTER(S)

BROTHER (S)

TOBACCO USE

CIGARETTES	PKG./DAY
CIGARS	#/DAY
PIPE	#/DAY

10035466OO

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996

LENOX HILL HOSPITAL

100 EAST 77TH STREET / NEW YORK, N. Y. 10021

Founded 1857

October 28, 1974

Frederic W. Nordsick, Ph.D.
Associate Research Director
The Council for Tobacco
Research - U.S.A., Inc.
110 East 59th Street
New York, N.Y. 10022

Dear Dr. Nordsick,

Here is my response to criticisms raised about my research proposal application, no. 996.

Comment #1: Although pulmonary resections are invariably associated with underlying pulmonary disease, the tissues were taken from grossly uninvolved areas. A moment's reflection will show that if indeed inorganic materials known to be carcinogenic or to otherwise promote pulmonary disease are found as proposed, that such findings are of immense importance depending upon the type of exposure subsequent individual history reveals. If absolutely necessary, one or two specimens could be obtained from accident victims from the medical examiners office, although this is administratively complicated.

Comment #2: If the patient's chart is inadequate, a questionnaire could be sent to the patient or his survivors to provide epidemiological information.

Division of Labor: The technician will embed, make thin and thick sections, stain, print negatives and otherwise provide essential assistance in performing the work of the proposal. The principal investigator will direct the technician, take orientation transmission electron micrographs as needed at Lenox Hill Hospital, advise the analytical microscope engineer where to take the spectrographs on the tissues of interest, collect the instrument data and collate it with epidemiological data as it is gathered. It is assumed that the principal investigator could operate the analytical microscope himself if such were available in the institution.

Travel funds cannot be broken down further in that experience must show how many trips will be required to develop a "working mode" for individual cases.

I hope these comments help to clarify the approach for accomplishing the goals of the proposal.

Sincerely yours,

John A. Terzakis, MD.

John A. Terzakis, M.D.

Distr. 11/1/74 LB

JAT:dg
10/28/74

bcc (by CTR) Drs. Hockett, Gardner, Sommers

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[redacted]

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 23 1974

Application for Research Grant
(Use extra pages as needed)

Date: July, 1974

1. Principal Investigator (give title and degrees):

John A. Terzakis, M.D.
Associate Pathologist

2. Institution & address:

Lenox Hill Hospital
100 East 77th Street
New York, N.Y. 10021

3. Department(s) where research will be done or collaboration provided:

Pathology

4. Short title of study:

Inorganic bronchial depositions

5. Proposed starting date: 1 Dec. 74

6. Estimated time to complete: 3 years

7. Brief description of specific research aims: A: To identify inorganic materials of atomic number greater than 10 and mass greater than 10-17 gm/cc in the cells of the bronchial mucosa of humans having undergone thoracotomy for various lung diseases.

B: To ultrastructurally localize such inorganic materials in these tissues for correlation with sites of known intracellular function.

C: To compare the association of such inorganic materials which occur together in the same cell for inferences regarding the source of these materials.

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8. Brief statement of working hypothesis: The various etiologic considerations for lung carcinoma and other respiratory diseases include atmospheric pollution, occupational causes and smoking, all of which deal with materials taken in by the human host. Of the numerous materials breathed in by humans, the potential organic agents are difficult to identify in tissues although of great importance. However, inorganic material can be so analyzed by x-ray microanalysis and ultrastructurally localized by concommitant observation in the scaning-transmission mode of an analytical electron microscope. Thus, one instrument performs two important tasks simultaneously. The data gained will serve for comparison with existing hypotheses of inorganic materials in the genesis of lung diseases (1-9). In this regard, Lewis et al (1-3) have studied cadmium levels in cadaver tissues and noted an increase in cadmium content in smokers versus non-smokers. My own preliminary findings using the method proposed has identified chromium, iron, cadmium, silicon, calcium, sulphur and a trace potassium in cytoplasmic granules of ciliated bronchial mucosa cells of a patient with bronchiectasis, chronic bronchitis and chronic non-specific pneumonia. These data bear on the protective functions of the human bronchial epithelium. Of course the moving blanket of mucin swept by cilia has long been recognized as a first line of defense against foreign materials. However, this preliminary work suggests that the ciliated cells participate in phagocytic activity commonly attributed to connective tissue macrophages or leucocytes.

9. Details of experimental design and procedures (append extra pages as necessary)

The tissues are available from 26 patients who had surgical resection at Lenox Hill Hospital. They exist in two forms: (i) tissue already fixed and embedded in plastic according to conventional techniques of electron microscopy, (ii) tissue from the same cases fixed in glutaraldehyde only which will be processed without use of osmium tetroxide, thus eliminating a material of considerable x-ray scattering ability. The embedded tissues will be cut on the ultramicrotome at a red reflection thickness and sections placed on nylon grids to eliminate scatter noted with metallic type grids. The principal investigator will supervise the examination of the material at the laboratories of the analytical microscope company, e.g. JEOLCO of Medford, Massachusetts. This is necessary to insure proper correlation of x-ray data with ultrastructural localization in the cells of interest. If possible, standards will be constructed for semi-quantitization. Conventional dark-room techniques will be utilized for printing of electron micrographs and spectrographs. Observations on the sectioned material will also be made utilizing the transmission electron microscopes presently available in our laboratory. From the data collected graphs will be constructed relating elements noted with location in bronchial tree, age of patient, smoking habits of patient, industrial exposure and location of patient's home. Insights regarding the location of inorganic material in mucosal cells will be explored to see if the contention that ciliated bronchial cells constitute a second line of defense against foreign materials can be verified.

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Selected References cited in #8:

1. Lewis, G.P., W. J. Jusko, L.L. Coughlin and S. Hartz, "Contribution of cigarette smoking to cadmium accumulation in man", Lancet (1) 291-292, 1972.

2. Nandi, M., D. Slone, H. Jick, S. Shapiro and G. P. Lewis, "Cadmium content of cigarettes", Lancet (2) 1329-1333.

3. Lewis, G. P., W. J. Jusko, L.L. Coughlin and S. Hartz, "Cadmium accumulation in man: Influence of smoking, occupation, alcoholic habit and disease" J. Chron. Dis. 25: 717-726, 1972.

4. Morgan, J.M., Burch, H.B., Watkins, J.B. "Tissue cadmium and zinc content in emphysema and bronchogenic carcinoma", J. Chron. Dis. 24: 107-110, 1970.

5. Sundermann, F.W., Donnelly, A.J., West, B. & Kinkaid, J.F., "Nickel poisoning. IX. Carcinogenesis in rats exposed to nickel carbonyl". Arch. Industr. Hlth 20: 36-41, 1959.

6. Wagoner, J.K., Archer, V.E., Carroll, B.E., Holoday, D.A. & Lawrence, P.A. "Cancer mortality patterns among U.S. uranium miners and millers, 1950 through 1962." J. Nat. Cancer Inst. 32: 787-801, 1964.

7. Bidstrup, P.L. and R.A.M. Case. "Carcinoma of the lung in workmen in bichromates-producing industry in Great Britain". Brit. J. industr. Med. 13: 260-264, 1956.

8. Bonser, G.M., Faulds, J.A. and Stewart, M.J. "Occupational cancer of urinary bladder in drystuffs operatives and of lung in asbestos textile workers and iron ore miners." Amer. J. Clin. Path. 25: 126-134, 1955.

9. Holland, R.H., Acevedo, A.R., & Clark, D.A. "The arsenic content of bronchial mucosa and submucosa in man. A comparison of specimens from lung cancer victims and control tissues". Brit. J. Cancer 14: 169-172, 1960.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Analytical electron microscope
JEOLCO
477 Riverside Avenue
Medford, Massachusetts 02155

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

#12: Biographical sketch: R, Bridgeport, Conn.: A.B. - Columbia College, 1957; M.D. - N.Y.U. School of Medicine, 1961; internship - Montefiore Hospital, 1961-1962; instructor in anatomy - N.Y.U. School of Medicine, 1962 - 1964; assistant professor of anatomy - N.Y.U. School of Medicine, 1964-1965; captain, U.S. Army Medical Corps - Walter Reed Army Institute of Research, 1965 - 1967; resident in pathology - Delafield Hospital, 1967-1968; resident in pathology - Lenox Hill Hospital, 1968-1969; assistant adjunct pathologist - Lenox Hill Hospital 1969-1970; adjunct pathologist - Lenox Hill Hospital, 1971-1973; associate pathologist - Lenox Hill Hospital, 1973-present; clinical assistant professor of pathology - Columbia University, 1967 - present; board certified in anatomic pathology - 1970; New York State medical license #089775; New Jersey medical license #28828; R

R

#13: Publications: (5)

✓ "Trimethoprin effect in Plasmodium gallinaceum oocysts", J.A. Terzakis, J. Parasitol. 57: 997, 1971.

✓ "Neurosecretory-appearing cells of human segmental bronchi", J.A. Terzakis, S. C. Sommers, & B. Andersson, Lab. Investigat. 26: 127-132, 1972.

✓ "Virus-like particles and sporozoite budding", J.A. Terzakis, Proc. Helmintholog. Soc. Wash. 39: 129-137, 1972.

"The mitochondria of pre-erythrocytic Plasmodium berghei", J.A. Terzakis, J.P. Vanderberg and R. M. Hutter, J. Protozool. 21: 251-253, 1974.

"X-ray microanalysis of hepatic thorium depositions", J.A. Terzakis, S.C. Sommers, R.W. Snyder, & M. Sabbath, Arch. Path. 1974.

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R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional: (give % time of investigator(s) even if no salary requested)	% time	Amount
John A. Terzakis, M.D.	25	
Technical		
Research Technician (to be recruited)	25	
Secretary (Miss D. Gordon)	4.543/hr	
Sub-Total for A		
B. Consumable supplies (by major categories)		
Chemicals		250
Glassware		250
Photographic supplies		250
Electron microscope accessories		250
Sub-Total for B		1,000
C. Other expenses (itemize)		
Travel		900
Miscellaneous		100
Sub-Total for C		1,000
Running Total of A + B + C		13,200
D. Permanent equipment (itemize)		
Sub-Total for D		13,200
E. Indirect costs (15% of A+B+C)		1,980
Total request		15,180

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2		1000	1000	-	1980	15,180
Year 3		1000	1500	-	2055	15,755

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Mailing address for checks

Principal Investigator

Typed Name John A. Terzakis, M.D.

Signature John A. Terzakis, MD Date 12/dec 74

Telephone R R
Area Code Number Extension

Responsible officer of institution

Typed Name Donald H. Black

Title Vice President

Signature [signature] Date 7/19/74

Telephone R R
Area Code Number Extension

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Source: https://www.industrydocuments.ucsf.edu/docs/mqdm0000